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Pathogenesis of mucosal disease and acute bovine viral diarrhea

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Pathogenesis of mucosal disease and acute bovine viral diarrhea

by

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GENERAL INTRODUCTION

Bovine viral diarrhea (BVD) virus, a pestivirus, is a ubiquitous bovine pathogen. There are two viral biotypes, noncytopathic [59,60,148] and cytopathic.[58,81] Two interacting viral-host factors involved in pestiviral disease are viral biotype and host immune response. Both factors play a role in the diverse clinical syndromes associated with BVD viral infection of cattle. Postnatal noncytopathic or cytopathic viral infection of normal nonpregnant seronegative susceptible cattle causes subclinical or mild self-limiting clinical disease with transient immunosuppression followed by seroconversion.[2,18,54,118] Infection of susceptible pregnant cattle with noncytopathic or cytopathic virus leads to fetal infection with several possible outcomes. Fetal infection at a vulnerable stage of organ development may result in fetal death or birth of a congenitally malformed calf.[7,71,72,129] Fetal infection with noncytopathic virus early in gestation before the onset of immunocompetence may result in birth of a malformed or clinically normal persistently infected calf.[84,92,140] Congenital infection with noncytopathic or cytopathic virus after the onset of fetal immunocompetence may result in birth of a malformed or clinically normal calf free of BVD virus and with precolostral antiBVD viral neutralizing antibodies.[84,102,154] A persistently infected individual superinfected postnatally with cytopathic virus may develop fatal mucosal disease or chronic BVD.[23,31]

Clinical syndromes that have been experimentally reproduced include acute cytopathic viral infection in postnatal cattle;[77,95,96] fetal infection with abortion, congenital anomalies and persistent

infection;[5,17,28-30,47,92,134] and mucosal disease.[22,23,31] Bovine viral diarrhea viral antigen localization has been well documented in cells of the lymphoid, gastrointestinal, integumentary and central nervous systems of cattle with persistent infection and mucosal disease.[5,6,9,10,46,94] Aspects of the pathogenesis of acute infection that need clarification are lesions and sites of BVD viral antigen distribution induced by noncytopathic and cytopathic viral infection of cattle known to be free of BVD antibody and virus. Pathogenic aspects of mucosal disease that warrant investigation are serologic responses of dual infected cattle to their own noncytopathic and cytopathic viruses and cellular distribution of BVD viral antigen in exocrine and endocrine glands of dual infected cattle.

The objectives of this study were i) to compare the lesions and sites of BVD viral antigen localization in cattle with induced acute infection by noncytopathic and cytopathic BVD virus; ii) to compare lesions and sites of BVD viral antigen localization in cattle with induced and spontaneous mucosal disease and spontaneous chronic BVD; iii) to determine the serum antibody responses of cattle with induced mucosal disease and with spontaneous mucosal disease and chronic BVD to their homologous noncytopathic and cytopathic viruses.

This dissertation is presented in the alternate format and consists of two scientific papers written for publication in refereed journals. The format used is that of Veterinary Pathology. A literature review precedes the first paper. The first paper has been submitted to Veterinary Pathology and the second paper will be submitted to American Journal of

Veterinary Research or to Veterinary Pathology. A list of references is at the end of each paper. A general summary and discussion follows the second paper. Literature cited in the general introduction, literature review, and general discussion and summary is at the end of the dissertation.

The National Animal Disease Center - National Veterinary Services Laboratories' Animal Care and Use Committee and The Iowa State University Committee on the Use of Animals in Research reviewed this project and concluded that the animal subjects were treated humanely in all procedures involved in this research.

The Ph.D. candidate, Catherine L. Wilhelmsen, was the principal investigator for each study.

REVIEW OF LITERATURE

Introduction

Bovine viral diarrhea (BVD) is a widespread, economically important disease of cattle, first described in 1946.[101] Recent reviews[2,18,32,54,118] describe several disease syndromes. The most common is an acute subclinical to clinically mild self-limiting disease which is accompanied by transient immunosuppression. Acute infections of pregnant cattle result in early reproductive failure, abortion, stillbirths, congenital defects and birth of normal appearing calves that are seropositive for antibodies to BVD virus and virus-free or seronegative and persistently viremic. Persistently viremic calves shed virus for life, serving as a source of infection to herdmates and offspring. Some persistently infected cattle may be chronically unthrifty and some may later develop acute severe fatal disease (acute mucosal disease) or chronic wasting disease (chronic mucosal disease or chronic BVD).

Virology and Molecular Biology

The cause of these disease syndromes is an enveloped single strand positive sense RNA virus classified as a Pestivirus in the nonarthropod-borne Togaviridae.[153] Pestiviruses are likely to be reclassified as Flaviviridae because of molecular similarities between pestiviruses and flaviviruses,[38,41] e.g., a single open reading frame, no subgenomic RNA and absence of a polyadenylated tail on the three prime end of the virion RNA.

The genomes of two BVD viral isolates have been cloned and sequenced.[40,123] Both are about 12.5 kilobases. A genomic map of one of the viruses [39] indicates viral proteins of the following sizes: a 20 kilodalton (kD) polypeptide; a putative 116 kD glycoprotein which gives rise to 62 and 53 kD glycoproteins, the former a precursor for the 48 and 25 kD glycoproteins; a 125 kD polypeptide from which 54 and 80 kD polypeptides may be derived; and a 133 kD polypeptide that may be the precursor for 58 and 75 kD products.

Two regions of the genome potentially code for polypeptides of 30 and 37 kD, but polypeptides have not been identified which correspond to these regions.[41] The expressed polypeptides or their proposed derived products essentially correspond to virally induced proteins found in BVD viral infected cell cultures.[45,49,50,89,108,116,117] The exception is a variable 32 to 38 kD protein found in infected cell cultures but not synthesized by the expression vector system[41] and not yet established as virus-encoded.

There are two biotypes of BVD virus, noncytopathic (NCP) [58,81] and cytopathic (CP) virus,[59,60,148] each with numerous representatives difficult to differentiate serologically. Monoclonal antibody panels indicate that antigenically related groups of BVD viruses contain both biotypes.[24] The variation in biotype appears to have a molecular basis. All BVD viral isolates examined so far[49,50,108,117] have a 115 to 120 kD protein, but only in CP isolates is this protein processed posttranslationally to an 80 kD protein. The 80 kD protein is related to the 115 to 120 kD protein by tryptic peptide mapping.[117] Monoclonal antibodies that react with the 115 kD protein also react with the 80 kD

protein.[97] The functions of the 115 and 80 kD proteins are unknown. Monoclonal antibody panels indicate that the 53 to 58 kD glycoprotein contains multiple epitopes involved in virus neutralization.[24,48] The functions of the other proteins are unknown.

The physical properties of BVD virus are well characterized.[153] Viral infectivity remains stable over a wide pH range (pH 6 to 9), but infectivity is inactivated by heating to 56 C for at least one hour and by exposure to ultraviolet light and detergents. The virus is sensitive to trypsin.

The in vitro growth characteristics of BVD virus vary with biotype. Cell cultures persistently infected with NCP BVD virus undergo earlier senescence than uninfected cells.[43] Continuous passage of persistently infected cells in the presence of homologous antibody[43] or alpha interferon[11] will not eliminate infection. In cell cultures infected with CP and NCP BVD viruses, NCP virus interferes with production of cytopathic effect (CPE) by the CP virus.[61] Cytopathic effect is characterized by vacuolation, rounding and detachment of infected cells.[59] The exact mechanism by which BVD virus induces CPE is not understood. Kinetic growth studies of cell cultures infected with NCP or CP BVD virus show the lag phase to last six to eight hours post inoculation (PI) followed by an exponential phase that lasts until 12 hours PI.[99] The cell surface receptor for BVD virus has not been identified.

Ultrastructure

Recent studies indicate BVD virions to be pleomorphic, roughly circular 40 to 60 nanometer diameter enveloped electron dense particles.[12,13,37,63] A central core of 20 to 30 nanometers in diameter is also described.[37,63] Viral replication takes place in the cytoplasm with budding of virions into cytoplasmic vesicles, possibly modified endoplasmic reticulum[37,63] or Golgi network[13] or autophagosomes.[13] Budding of viral particles from the plasma membrane has not been observed.

Acute Bovine Viral Diarrhea

This syndrome is acute infection in seronegative immunocompetent cattle in post natal life.[2] The most common syndrome, it is characterized by inapparent infection or mild self-limiting clinical disease. Recovery is marked by seroconversion with clearance of the virus from the body. Serologic surveys indicate that between 60% and 90% of cattle have serum neutralizing antibodies to BVD virus,[21,66] suggesting that inapparent or mild acute BVD is widespread. Transmission of BVD virus is by ingestion or inhalation of material contaminated by infected oculonasal discharges, saliva, urine and feces.[54] Spread by fomites is less important.[54] In acute infection of susceptible cattle, there is a five to seven day incubation period[20,53] followed by onset of pyrexia, hyperpnea, anorexia and leukopenia.[20,53,100] Occasionally, there is diarrhea and oculonasal discharge. The viremia can persist up to 15 days after exposure, followed by the rapid appearance of serum neutralizing antibodies.[20,100] During acute infection, virus can be isolated from

urine and most organs, [95,96] blood and nasopharyngeal secretions,[100] and semen.[104] Acutely infected bulls may have lowered semen quality, manifested as reduced sperm density and motility and increased sperm abnormalities.[104]

Lesions reported in pathogenesis studies of experimental post natal acute CP BVD viral infection in cattle[77,95,96] were multifocal hemorrhages; hemorrhagic enteritis; oral, ruminal and turbinate erosions; necrosis and depletion of lymphoid tissues; arteritis, gliosis and perivascular cuffs in the brain. Bovine viral diarrhea viral infection of the respiratory tract may be prolonged.[95] The aforementioned studies were conducted before the extent of contamination of cell culture reagents with NCP BVD virus was well known.[86] It is unclear whether experimental viral inocula used in these early studies were free of NCP BVD virus.

Fetal Infection

Infection of seronegative, susceptible cows at or soon after breeding may result in early reproductive failure.[62] Venereal transmission of BVD virus may occur with natural service.[91] Embryo transfer is a theoretical route of BVD viral transmission,[114] but the effect of BVD virus on embryos has not been thoroughly investigated. Infection of the fetus from fertilization to four months in gestation may result in fetal resorption; abortion of fresh or autolyzed or mummified fetuses;[36,73,129] stillbirths with or without congenital defects[7,17] and stunted growth.[149] Lesions in natural and experimental cases of bovine fetal infection with CP or NCP BVD virus between fertilization and

the end of four months gestation include cerebellar degeneration with granuloprival hypoplasia;[7,30,47,144] hypomyelination;[16,47,140] microencephaly;[7,47] hydranencephaly;[144] encephalitis with perivascular mononuclear cells;[16,29] porencephaly;[7,30] internal hydrocephalus;[7,47,144] thymic hypoplasia or atrophy;[7,16,47] intrauterine growth retardation;[47] arthrogryposis;[7] brachygnathism;[17] and alopecia or hypotrichosis with follicular hypoplasia.[73]

Fetal infection with NCP BVD virus within the first four months (before 125 days) of gestation may result in failure of the fetus to produce specific neutralizing antibody and clear the virus. Such a calf may be born seronegative and persistently viremic.[92] The calf may grow and survive for several years with its persistent viral infection, shedding virus for life and serving as a source of infection for seronegative, susceptible cattle. Offspring of persistently infected cows also are persistently infected.[53]

Fetal infection with NCP or CP BVD virus between five and seven months of gestation may result in abortion of fresh or mummified fetuses;[129,134] stillbirths with or without congenital defects;[17] or birth of normal seropositive calves.[102] The onset of fetal immunocompetence usually occurs during this period. In a field study, calves presumably infected in utero with BVD virus after their dams were vaccinated with modified live virus vaccine after 190 days of gestation were born with precolostral neutralizing antibodies to BVD virus.[102] Experimental in utero infection of fetuses between 120 and 165 days of

gestation resulted in fetal and maternal seroconversion within three weeks PI and failure to isolate virus from fetal tissues.[5,14] Lesions in natural and experimental bovine fetal infection with NCP or CP virus between five and seven months of gestation include cerebellar degeneration or hypoplasia;[5,17,29,36,71,72,134,145,151,154] hypomyelination;[154] hydranencephaly;[145] encephalitis with perivascular infiltrates;[29] porencephaly;[29,154] internal hydrocephalus;[145] focal hemorrhages;[29,134] cataract;[17,71,72,129,134] retinal degeneration;[17,28,71,134] optic nerve hypoplasia;[17,134] thymic hypoplasia;[5] precocious development of secondary lymphoid tissues;[5] brachygnathism;[129,134] hyperkeratosis of the skin;[5] and necrosis of cells in the skin,[5,36] mucous membranes[5,71,72,151] and lung.[36]

Fetal infection with NCP or CP virus from the end of the seventh month of gestation to parturition may result in fetal production of a neutralizing antibody response with elimination of the viral infection. In a calf that survives to birth, a precolostral serum sample from the neonate may contain BVD viral neutralizing antibodies.[84,102]

Persistent Infection

In the field, a persistently infected bovine introduced into a herd of seronegative, BVD virus-free pregnant cows can transmit NCP BVD virus to the cows, resulting in maternal and fetal NCP BVD viral infection. The cows will have acute subclinical or mild clinical BVD followed by the appearance of specific neutralizing antibodies. Depending on the time in gestation, any or all of the effects of fetal infection may be manifested, including birth of persistently infected calves.[22,53,140] Persistently

infected females produce persistently infected calves, establishing maternal families of persistently infected cattle.

Reported rates of persistent infection range from 0.4% to 8.1%,[21,68,135] but sampling of populations was nonrandom. Persistently infected cattle may be clinically normal[91] or malformed[3] or stunted and unthrifty.[54,118] Besides congenital anomalies, persistently infected cattle may have mesangioproliferative glomerulonephritis[46,67] and lymphocytic encephalitis.[46] Virus can be isolated from blood, ocular and nasal secretions and a wide range of tissues.[3,42,91] The semen of persistently infected bulls may be of poor quality.[91,125] The virus may be transmitted by infected semen. Susceptible heifers bred to a persistently viremic bull did not conceive until they seroconverted to BVD virus.[91]

Bovine viral diarrhea viral antigen can be demonstrated in most organs of persistently infected cattle. Sites of intracytoplasmic BVD viral localization include neurons of the central nervous system;[46,56] keratinocytes of the stratum basale and stratum spinosum of the skin and upper digestive tract;[9,10] salivary acinar and bronchiolar epithelial cells;[9] renal tubular cells and glomeruli;[9,46] lymphocytes, macrophages and dendritic cells in lymphoid tissues;[9] intestinal crypt cells;[9,46] testicular tubular cells[46] and peripheral blood mononuclear cells.[8,15] A double labelling experiment showed T lymphocytes, B lymphocytes, monocytes and null cells to contain BVD viral antigen.[8] Sixty percent of BVD viral antigen containing T lymphocytes were CD8 positive and 40% were CD4 positive.[8]

Bovine viral diarrhea virus may have a predilection for T lymphocytes. Cell sorting of peripheral blood mononuclear cells from persistently infected cattle revealed an overall infection rate of 4.4% of all mononuclear leukocytes with a 5.4% infection rate in a lectin labelled T lymphocyte enriched fraction and a 2.1% infection rate in an antiglobulin labelled B lymphocyte enriched fraction.[26]

Mucosal Disease

Severe clinical disease characterized by hemorrhagic diarrhea is experimentally induced in persistently infected cattle by superinfection with CP BVD virus.[22,23,31] Only certain complementary pairs of CP and NCP BVD viruses induce mucosal disease.[22] Clinical signs of induced acute mucosal disease[23] resemble those of spontaneous mucosal disease.[2] Signs in induced disease are anorexia, excessive salivation and lacrimation and diarrhea with blood and mucus beginning 10 to 26 days after inoculation of the CP BVD virus.[23] The diarrhea becomes intermittent and watery and is accompanied by tenesmus. After onset of clinical signs, dehydration and loss of body weight are rapid.

Lesions of induced mucosal disease resemble lesions of spontaneous field cases of mucosal disease and post vaccinal disease. Gross lesions of induced mucosal disease[23] include thickening of the cecum and proximal colon with focal ulcerations; erosions and ulcers of the esophagus, abomasum, jejunum, ileum and rectum; elongated ulcers over ileal Peyer's patches; gall bladder mucosal erosions; epicardial and ileal serosal hemorrhages. Microscopic lesions of the alimentary tract include

epithelial necrosis and sloughing; lymphocytic infiltration of the lamina propria; necrosis of Peyer's patches with submucosal prolapse and cystic dilatation of intestinal glands. Microscopic lesions in other systems include renal glomerular hypercellularity and thickening of the basement membrane; lymphoid cell accumulation in hepatic triads; pulmonary interstitial and interlobular emphysema with fibroplasia and excessive bronchus associated lymphoid tissue.[23] Similar lesions in field cases of mucosal disease[35,64,69,74,119,120,133,147] and in post vaccinal disease[105] occur in the enteric, lymphoid, circulatory, respiratory and integuementary systems. Lesions are erosions, ulcers and cystic changes of the gastrointestinal tract; edema, hemorrhages, lymphocytic necrosis and depletion of lymphoid tissues; hemorrhages and periateritis; catarrhal to mucopurulent rhinitis with erosions and ulcerations; pulmonary emphysema and proliferative dermatitis. Other lesions that may occur in mucosal disease are amyloidosis[143] and perivascular lymphocytic cuffs in the brain.[76]

Immunohistochemical studies of tissues from cattle with spontaneous mucosal disease[6,94,115] demonstrate BVD viral antigen localization similar to that in persistent infection.[9,10,46,56] Additional sites of BVD viral antigen in mucosal disease are intestinal intramural ganglia,[109] corneal epithelium and nasal mucosa.[94]

Chronic Bovine Viral Diarrhea

Instead of becoming moribund soon after onset of signs of mucosal disease, some cattle become chronically afflicted with what is termed

chronic BVD or chronic mucosal disease[2] or chronic wasting disease.[32] The clinical signs are inappetence, weight loss, progressive emaciation, rough haircoat, continual or intermittent diarrhea, chronic bloat, nasal and ocular discharge, alopecia, sporadic mucosal erosions and chronic lameness due to laminitis and interdigital necrosis.[2,25,70,83,139] Hematologic changes are pancytopenia, leukopenia and lymphopenia.[2,25,98] Chronic BVD cases may be complicated by secondary bacterial infections. Cattle with chronic BVD ultimately die of severe debilitation.

Lesions of chronic BVD differ somewhat from those of classical mucosal disease. Chronic BVD lesions are healed erosions of the upper alimentary tract,[25] dilation of colonic glands with little or no periglandular lymphoid tissue,[25] thymic lymphocytic depletion,[98] hypoplasia of bone marrow hematopoietic cells,[98] splenic hemosiderosis[98] and mild interstitial pneumonia with bronchial metaplasia[98] or chronic fibrosing pneumonia.[82]

The viral pathogenesis of chronic BVD may be similar to mucosal disease. Cattle with field cases of chronic BVD have congenital persistent NCP BVD viral infection, but CP BVD virus is not always isolated.[25,83,90] Chronic BVD has not been induced by CP BVD viral superinfection of persistently infected cattle.

Immunology

Bovine viral diarrhea virus exerts immunosuppresive effects in vitro. Cultivated macrophages and lymphocytes from peripheral blood can be

infected by, and support replication of, BVD virus.[146] Bovine peripheral blood monocytes exposed to BVD virus in vitro have a markedly depressed response to chemotactic stimuli.[75] Mitogen induced proliferation of bovine mononuclear cells[87] and mitogen induced bovine splenic plasma cell development[1] are inhibited by BVD viral infection. Bovine viral diarrhea viral infected fetal lung culture cells release immunosuppressive substances, possibly prostaglandins, capable of inhibiting mitogen induced proliferation of cells.[87] Bovine viral diarrhea induces interferon[11,126] and prostaglandin secretion may be mediated by interferon production[87].

Acute experimental infection of cattle results in transiently decreased numbers and impaired function of leukocytes. The transient leukopenia lasts for about seven days post inoculation (PI)[20,55] with decreased absolute numbers of B lymphocytes and T lymphocytes,[20] and a reduction in both major T lymphocyte subsets (CD4 and CD8),[55] followed by the appearance of BVD viral neutralizing antibodies by 17 days PI.[20] Inhibition of mitogen induced lymphocyte blastogenesis is an effect of acute experimental infection with virulent BVD virus[110,122] or with modified live virus vaccine.[131] Acute BVD viral infection may interfere with bacterial clearing mechanisms of the body, resulting in endogenous bacteremia.[122] Inoculation of virulent virus or modified live virus vaccine can lead to decreased circulating neutrophils and to prolonged (up to a month long) suppression of neutrophil iodination, which is an in vitro functional test of the myeloperoxidase, hydrogen peroxide halide antibacterial system.[131,132] Administration of modified live virus

vaccine also results in suppressed antibody dependent cell-mediated cytotoxicity (ADCC) by neutrophils.[131]

Experimental infections of cattle with BVD virus and other infectious agents suggest that BVD virus may exert a synergistic effect on the other agents. Mixed infections of the respiratory tract involving BVD virus and infectious bovine rhinotracheitis (IBR) virus[112] and Pasteurella haemolytica[85,111,113] and of the gastrointestinal tract involving BVD virus with Salmonella dublin and S. typhimurium[155] have been studied. Cattle dually infected with BVD virus and other agents had more severe lesions or more extensive tissue infection than cattle solely infected with BVD virus. Use of modified live virus BVD vaccines in cattle entering the feedlot may also induce immunosuppression, possibly leading to increased mortality.[88]

Immunologic studies show that peripheral blood leukocytes from persistently infected cattle are functionally impaired. The mitogen stimulated proliferative response of peripheral blood mononuclear cells from persistently infected cattle is less than that of normal control cattle.[79,130] Depleting mononuclear cells of suppressor cells increases the mitogen stimulated proliferative response of the remaining mononuclear cells.[79] Ingestion of Staphylococcus aureus by neutrophils is impaired in persistent infection. However, iodination and ADCC are unaffected.[130] Despite functional impairment of mononuclear cells, persistently infected calves are able to synthesize immunoglobulins of several isotypes (IgG1, IgG2, IgM) reaching the same total serum protein levels as normal healthy cattle by a year of age.[44]

Cattle with spontaneous or induced mucosal disease have depressed leukocyte counts and impaired leukocyte function, indicative of immunosuppression. Experimental inoculation of persistently infected cattle with CP BVD virus results in decreased total leukocyte count; decreased absolute lymphocyte count; drops in the percentage and absolute number of T lymphocytes, with lowest counts between seven and 11 days PI and before the onset of clinical disease.[23] Lymphocytes from cattle with field cases of mucosal disease respond poorly to mitogen stimulation.[137] Increased mitogen induced lymphocyte blastogenesis occurs when a suppressor cell subpopulation with a receptor for the Fc part of IgG is removed from the lymphocyte mixture.[78]

The nature of the immunological state of nonresponsiveness (tolerance) in mucosal disease is highly specific.[19,22,137] Neutralizing antibodies were not detected in several studies of natural persistent infection or spontaneous mucosal disease.[52,92,128,137] Yet, cattle in several studies produced antibodies in titers comparable to normal healthy cattle for many unrelated pathogens including IBR virus, parainfluenza 3 virus, parvovirus, rotavirus and Pasteurella haemolytica.[92,137] Some persistently infected cattle may have a depressed antibody response to bovine leukemia virus.[127] Persistently infected cattle experimentally inoculated with CP BVD viral isolates often produce neutralizing antibodies specific for the isolates administered and some, but not all, of these cattle may go on to develop fatal mucosal disease.[19,22,23,83,136]

Both the humoral and cellular immune systems are impaired in chronic BVD. Cattle with chronic BVD may have fewer surface immunoglobulin

containing (B) cells than normal cattle.[98] Lymphocytes from cattle with chronic BVD are less responsive to mitogen-induced blastogenesis than lymphocytes from normal cattle.[25,70]

Other Pestiviruses

Border disease virus (BDV) and hog cholera virus (HCV) have several features in common with BVD virus. All three pestiviruses are antigenically related.[93,103,106] Cytopathic and noncytopathic biotypes occur in HCV[80] and in BDV.[57,150] Like BVD virus, HCV[34,107] and BDV[149] can induce persistent viremia. A mucosal disease like syndrome has been reported in sheep with border disease.[57] The three pestiviruses have partially overlapping host ranges. Bovine viral diarrhea virus can infect swine[33,138,142,152] and sheep[57] and BDV can infect cattle[4] and pigs,[141] but HCV appears to infect only swine.[65]

PATHOGENESIS OF EXPERIMENTAL ACUTE BOVINE VIRAL DIARRHEA IN CALVES

Pathogenesis of experimental acute bovine viral diarrhea in calves

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ABSTRACT

Eight healthy calves were inoculated intranasally with noncytopathic (n=4) or cytopathic (n=4) bovine viral diarrhea (BVD) virus and were examined postmortem five or 12 days post-inoculation. The most frequent gross lesion was proximal colonic mural edema. Consistent microscopic findings were acute to subacute tracheitis, mild enterocolitis with edema, petechial hemorrhages of mesenteric lymph nodes with mild follicular lymphocytic depletion, and paracortical lymphoid hyperplasia. At necropsy, virus was recovered from six calves and neutralizing antibodies to BVD virus were detected in the other two calves. Immunohistochemical analysis of lymphoid tissues demonstrated a small, widely scattered population of mononuclear cells expressing BVD viral antigen.

INTRODUCTION

Biotypes of bovine viral diarrhea are classified as noncytopathic or cytopathic based on effects observed in infected cell monolayers.[16,17] Singly, or in combination, the two biotypes of BVD virus induce clinical disease involving lymphoid, enteric, respiratory, and reproductive systems.[1,32] The lesions best characterized are those associated with the rare and highly fatal mucosal disease and chronic BVD forms.[13,19,31,33,41] These diseases are sequelae of a persistent noncytopathic BVD virus infection established in the fetus during the first four months of gestation.[23,25] Clinical disease is induced by superinfection with the appropriate cytopathic BVD virus.[6,11]

The most common form of BVD is the clinically mild or inapparent acute infection, which is induced by either viral biotype. This form of infection results in immune depletion and immune suppression,[5,27,36] is a contributing factor in bovine respiratory disease,[28-30] and may adversely affect the developing fetus.[8-10,38,40,42] Descriptions of lesions induced by acute cytopathic BVD virus infection in healthy cattle include erosions, hemorrhages, and periarteritis in the alimentary tract, and edema and hemorrhages with lymphocytic necrosis and lymphoid depletion in lymph nodes and spleen.[21,26] Our purpose was to produce acute noncytopathic and cytopathic BVD viral infection, to characterize the lesions induced, and to use immunohistochemical techniques to identify sites of viral replication.

MATERIALS AND METHODS

Ten crossbred calves approximately six months of age were allotted into five groups of two calves each (group 1, calves 1 and 2; group 2, calves 3 and 4; group 3, calves 5 and 6; group 4, calves 7 and 8; group 5, calves 9 and 10; Table 1). Each group of calves was kept in separate enclosed pens at opposite ends of an isolation barn; calves receiving noncytopathic BVD virus were physically separated from calves receiving cytopathic BVD virus by a 10 meter zone. With one exception, all calves lacked serum neutralizing antibodies to BVD virus by standard microtiter methods.[35] The one calf having a neutralizing antibody titer to BVD virus at the 1:4 dilution of serum was assigned to the control group. All calves were free of BVD virus by viral isolation from serum and buffy coat. Baseline total and differential leukocyte counts on all calves were within normal limits.[20] On day zero, a serum sample was obtained from each calf, and eight calves were inoculated intranasally with approximately 10^8 cell culture infectious doses of noncytopathic BVD-TGAN virus (n=4) or cytopathic BVD-TGAC virus (n=4). The cytopathic virus had been purified by three successive plaque pickings and the noncytopathic virus had been purified by three successive passages at limiting dilution. On day 1 post-inoculation, the control calves (group 5, n=2) were anesthetized, exsanguinated, and examined postmortem. On days 5 and 12 post-inoculation, four calves receiving BVD-TGAN (groups 1 and 3, n=2) or BVD-TGAC (groups 2 and 4, n=2) were anesthetized, exsanguinated, and examined postmortem.

Table 1. Viral isolation, serology, gross lesions

Viral biotype[a]	1 NCP	2 NCP	3 CP	4 CP	5 NCP	6 NCP	7 CP	8 CP	9 C	10 C
Sex	MC	F	F	F	F	F	MC	MC	F	F
Days PI[b]	5	5	5	5	12	12	12	12	1	1
Viral isolation	+[c]	+	+	+	-	-	+	+	-	-
Serum titer	<1:2	<1:2	<1:2	<1:2	1:4	1:4	<1:2	<1:2	1:4	1:2
Mucosal thinning over Peyer's patches	-[d]	-	-	-	-	+	+	+	-	-
Mural edema of distal ileum	+	+	+	+	+	+	+	+	-	-
Mesenteric lymphadenopathy	+	+	-	-	+	+	+	+	-	-
Ileocecal valvular edema and erosions	-	+	+	+	+	-	+	+	-	-
Distal colonic mucosal petechiation	-	-	-	-	+	+	-	-	-	-

Mesenteric lymph node petechiation	-	-	+	+	+	+	+	+	-	-
Enlarged hemal nodes	+	+	-	+	-	-	+	-	-	-

[a]NCP = noncytopathic; CP = cytopathic; C = control.

[b]PI = post-inoculation.

[c]+ = virus was isolated; - = virus was not isolated.

[d]+ = lesion was present; - = lesion was absent.

At the time of necropsy, blood was collected for total and differential leukocyte counts, and virus and bacterial isolation. Serum was obtained for virus neutralization tests, which were performed using standard microtitration procedures.[35] Tissues collected at necropsy for viral isolation were proximal colon, perirectal lymph node, spleen, and thymus. Twenty percent tissue suspensions were inoculated onto bovine turbinate (BT) cell cultures, incubated at 37 C and observed daily for cytopathic effect (CPE). All inoculated cultures were tested for BVD viral antigen by the direct fluorescent antibody technique, using fluorescein conjugated bovine hyperimmune immunoglobulin. The BT cells and culture medium used for viral neutralization tests and viral isolation procedures were free of adventitious BVD virus and antibodies to BVD virus. For bacterial isolation, serum was inoculated onto sheep blood agar plates, incubated aerobically at 37 C for four days and observed daily for bacterial colonies.

Tissues collected and fixed in 10% neutral buffered formalin for microscopic examination were adrenal gland, abomasum, brain, cecum, distal colon, distal ileum, esophagus, eye, femoral bone marrow, first cervical spinal cord segment, gall bladder, gonad, heart, ileocecal valve, jejunum, kidney, liver, lung, mediastinal, mesenteric and retropharyngeal lymph nodes, pancreas, parotid salivary gland, pituitary gland, proximal colon, rumen, spleen, thyroid, thymus, tonsil, trachea, and urinary bladder. Tissues frozen unfixed in 2.5% methyl cellulose for immunohistochemical examination were distal ileum, lung, mesenteric lymph node, and proximal colon. Further processing of tissues for light microscopy was done by

standard methods. For immunohistochemistry, 6 μ thick cryosections were collected on poly-L-lysine-coated glass slides, air dried, and fixed in 100% chilled acetone (-20 C). Rehydration was done with Tris buffer,[39] which also was used for all dilutions and washes. After blocking with 0.5% horse serum in Tris buffer for 20 minutes at room temperature, tissue sections were coated with monoclonal antibody (MAB) to BVD virus[7] and incubated for 18 hours at 4 C in a humidified atmosphere. Peroxidase or alkaline phosphatase staining was done with biotinylated equine anti-mouse IgG and avidin-enzyme complex (Vectastain ABC Immunoperoxidase Elite Kit or Vectastain ABC Alkaline Phosphatase Kit, Vector Laboratories, Burlingame, CA). After immersion in the appropriate substrate, the tissue sections were post-fixed in formol saline, counterstained with hematoxylin, and dehydrated in increasing concentrations of alcohol. All tissues were reacted with at least 3 MABs[7] of known specificity for BVD-TGAC and BVD-TGAN viruses. Controls comprised known negative and positive samples (tissues from cattle with mucosal disease), replacement of MAB by serum supplemented cell culture medium, and substitution of specific MABs with nonspecific MABs.

RESULTS

All calves remained clinically normal except for a mild bilateral serous nasal discharge observed in two calves 12 days after inoculation with cytopathic BVD-TGAC virus. Compared to pre-inoculation values obtained for each calf, blood obtained before necropsy 5 days post-inoculation showed decreased leukocyte counts (25% or greater) for both calves given noncytopathic BVD-TGAN virus and essentially unchanged counts in the two calves given cytopathic BVD-TGAC virus. At 12 days post-inoculation, the leukocyte counts for all 4 calves were similar to or exceeded those obtained on day zero.

Subtle gross lesions were confined to the alimentary (Figs. 1, 2) and lymphoid systems. All eight calves had moderate proximal colonic mural edema approximately 25 cm distal to the ileocecal valve and had mild to severe swelling and edema of mesenteric lymph nodes. Six of 8 calves had cortical petechial hemorrhages in the mesenteric lymph nodes. Other gross lesions are listed in Table 1. Incidental gross lesions in individual calves were corneal scarring, pthisis bulbi, and chronic liver abscess.

Microscopic lesions were present in the respiratory, alimentary, and lymphoid systems. Inoculated animals had a mild acute to subacute purulent tracheitis, characterized by coagulative necrosis of mucosal epithelial cells and by mucosal and submucosal neutrophilic infiltrates (Fig. 6). In the intestinal tract of principals and controls, there were randomly distributed crypt abscesses and mild to moderate mucosal eosinophilic infiltrates. All inoculated calves had mild to moderate intestinal lymphangiectasia and separation of smooth muscle fibers,

Figure 1. Proximal colon, calf No. 5 (noncytopathic virus, 12 days post-inoculation). Mucosal edema overlying patch of submucosal lymphoid tissue

Figure 2. Proximal colon, calf No. 1 (noncytopathic virus, 5 days post-inoculation). Mucosal edema and hyperemia

Figure 3. Mesenteric lymph node, cryosection, calf No. 7 (cytopathic virus, 12 days post-inoculation). BVD viral antigen containing mononuclear cell within germinal center

Figure 4. Mesenteric lymph node, cryosection, calf No. 7 (cytopathic virus, 12 days post-inoculation). BVD viral antigen containing cell within medulla

Figure 5. Mediastinal lymph node, cryosection, calf with mucosal disease. Numerous BVD viral antigen containing cells within medulla

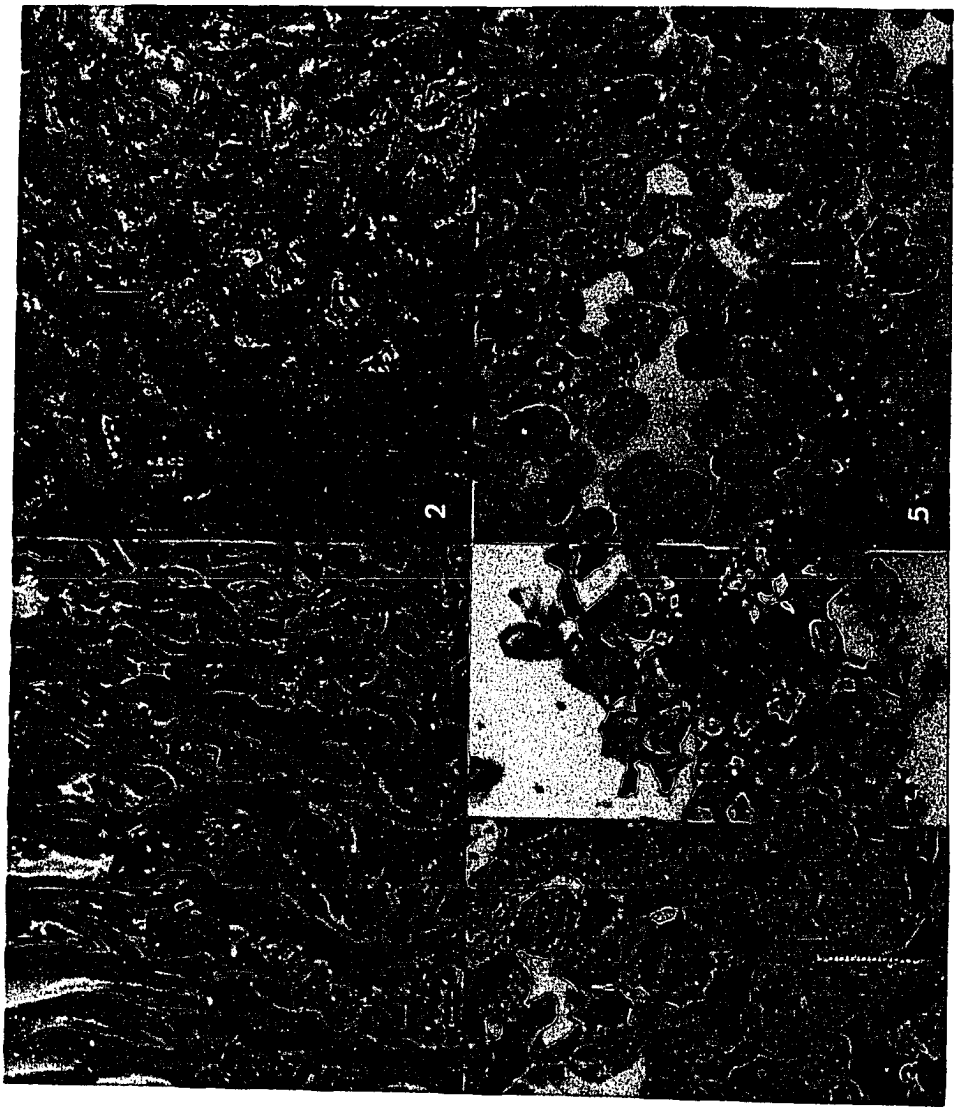
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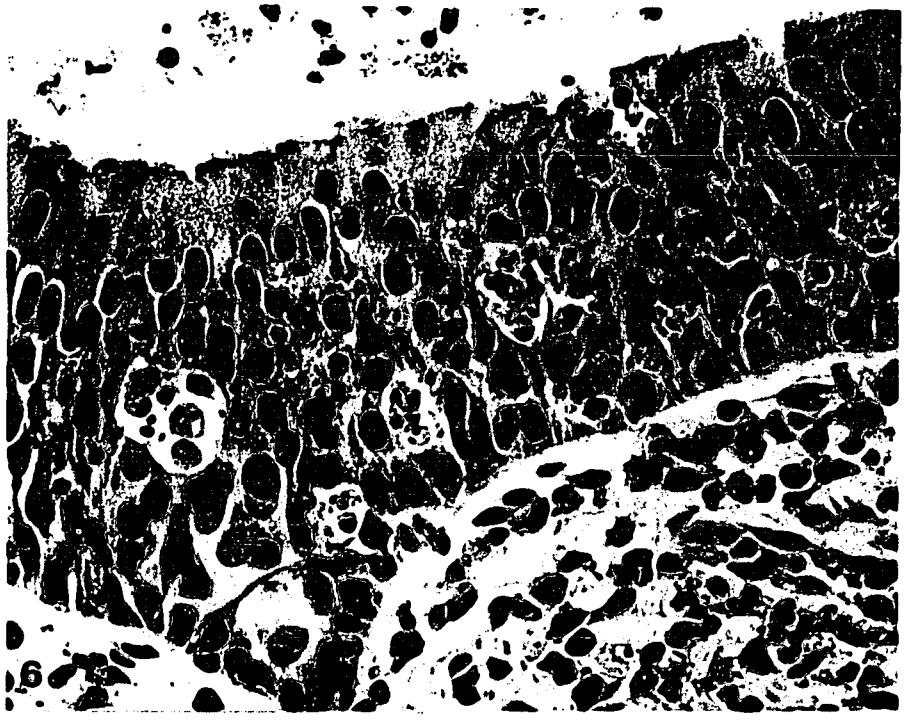
Figure 6. Trachea, calf No. 2 (noncytopathic virus, 5 days post-inoculation), HE. Necrotic epithelial cells, intraepithelial leukocytes, and submucosal inflammatory cell infiltrate

Figure 7. Proximal colon, calf No. 5 (noncytopathic virus, 12 days post-inoculation), HE. Peripheral vacuolation of ganglionic neurons within myenteric plexus



interpreted as edema. A few autonomic ganglion cells of ileal and colonic myenteric plexuses of three inoculated calves had mild peripheral cytoplasmic clearing or vacuolation (Fig. 7). Mild to moderate follicular lymphocytolysis characterized by pyknotic and fragmented lymphocytes and by macrophages bearing engulfed cellular debris was in small and large intestinal submucosal lymphoid nodules of all inoculated animals. There was consistent mild distension of subnodular lymphatic spaces. Two calves (Nos. 6 and 8, Table 2) had several mesenteric periarterial infiltrates of lymphocytes and macrophages. Mesenteric lymph nodes of principals had mild to moderate follicular lymphocytolysis (Fig. 8) and scattered intrafollicular globule leukocytes. Petechial hemorrhages, when present, were within follicles (Fig. 8) and paracortical areas. Paracortical (T cell dependent) areas in lymph nodes of all four calves at 12 days post-inoculation were populated by many small lymphocytes; by a lesser number of lymphoblasts, some in mitosis; and by a few macrophages with pale cytoplasm. Laminated mineralized bodies were randomly distributed in mesenteric lymph node follicles of two calves (Nos. 7 and 8, Table 2). Other microscopic lesions are summarized in Table 2. All principals and one control (No. 10) had a mild diffuse eosinophilic enterocolitis with coccidial meronts and gamonts within mucosal cells. Other incidental parasitic lesions in experimental and control animals were glossal and cardiac sarcosporidiosis and abomasal nematodiasis.

Immunohistochemical staining of ileum, colon, lung, and mesenteric lymph node revealed rare, widely distributed BVD viral antigen containing mononuclear cells in the gut-associated lymphoid tissue,

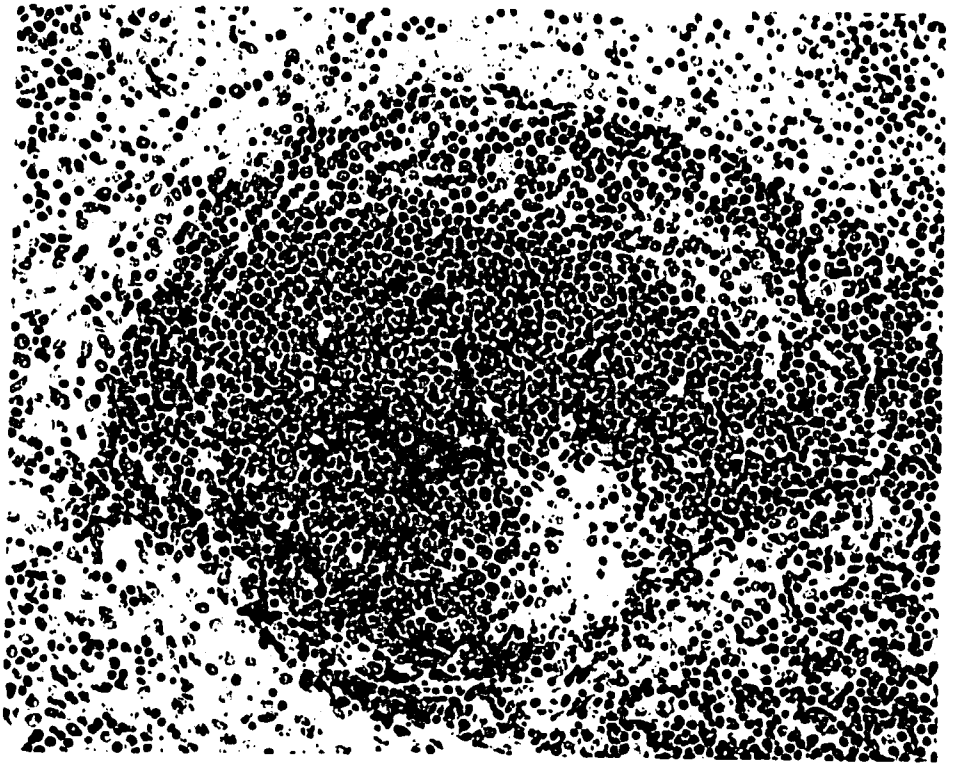
Table 2. Summary of microscopic lesions

	Calf Number									
	1 NCP	2 NCP	3 CP	4 CP	5 NCP	6 NCP	7 CP	8 CP	9 C	10 C
Purulent tracheitis	+[a]	+	NE[b]	+	+	+	NE	+	-	-
Mild eosinophilic bronchitis	-	-	-	-	+	-	-	-	-	-
Splenic lymphoid depletion	+	+	+	-	+	-	-	-	-	-
Vacuolation of myenteric ganglion cells	-	-	-	-	+	+	+	-	-	-
Mesenteric periarteritis	-	-	-	-	-	+	-	+	-	-
Mesenteric lymph node mineralization	-	-	-	-	-	-	+	+	-	-
Lymphoid hyperplasia of hemal nodes	+	-	+	+	-	+	-	-	-	-

[a] + = Lesion was present; - = lesion was absent.

[b] NE = Not determined.

Figure 8. Mesenteric lymph node, calf No. 6 (noncytopathic virus, 12 days post-inoculation), HE. Follicular lymphocytic depletion and hemorrhage



bronchus-associated lymphoid tissue and cortex, paracortex, and medulla of mesenteric lymph nodes (Figs. 3, 4) of one or more inoculated animals (Table 3). Autonomic ganglion cells containing BVD viral antigen were seen in the intestine of one calf (No. 7). Incompletely blocked granulocytic endogenous peroxidase activity[22] or nonspecific avidin binding to mast cells[12] present within the lamina propria of some sections of ileum and colon could not be differentiated from specific immunoreactivity. In contrast to tissues from the experimental calves, positive control tissues from cattle with mucosal disease had numerous BVD viral antigen containing cells (Fig. 5).

Bovine viral diarrhea virus of the appropriate biotype was isolated from one or more tissues from 6 of 8 calves. Virus was not isolated at 12 days post-inoculation from tissues of the two calves given noncytopathic BVD-TGAN virus. These calves had a serum neutralizing antibody titer of 1:4 to noncytopathic BVD-TGAN virus and a titer of less than 1:2 to cytopathic BVD-TGAC virus. The other six calves had a titer of less than 1:2 to both biotypes of BVD virus at the time of necropsy. Other viruses or bacteria were not isolated from the blood of any calf.

Table 3. Results of immunohistochemistry

	Calf Number									
	1 NCP	2 NCP	3 CP	4 CP	5 NCP	6 NCP	7 CP	8 CP	9 C	10 C
Lung	-[a]	-	-	+	-	-	-	-	-	-
Mesenteric lymph node	+	-	-	-	+	+	-	-	-	-
Distal ileum	-	+	-	+	+	-	+	-	-	-
Proximal colon	-	+	-	-	-	-	+	+	-	-

[a] + = BVD viral antigen containing cells were present; - = BVD viral antigen containing cells were absent.

DISCUSSION

The two viral biotypes used in this study induced similar gross and microscopic lesions in the lymphoid, enteric, and respiratory systems. The lesions induced in acute experimental BVD viral infection were frequently similar to, but always milder than those described in mucosal disease.[31,33,34] Unlike in mucosal disease, no cutaneous or upper alimentary tract lesions were seen in acute experimental BVD viral infection.

Lymphoid lesions of acute experimental BVD viral infection share some features with lymphoid changes described in field cases of mucosal disease.[13,33,41] Gross swelling and edema of mesenteric lymph nodes are found in both conditions. The mild follicular lymphocytolysis in mesenteric lymph nodes of acutely infected calves may be a lesser degree of the marked follicular lymphocytic depletion or exhaustion in mesenteric lymph nodes in cattle with mucosal disease.

Lymph node lesions in acute experimental BVD viral infection are assumed to be the result of viral infection. In three acutely infected calves (Nos. 1, 5, 6, Table 1) with enlarged, edematous mesenteric lymph nodes, viral antigen-bearing cells detected immunohistochemically within these nodes are interpreted as infected cells. Necrosis and lysis of follicular cells in mesenteric lymph nodes of all acutely infected calves may be due to direct viral damage, to host cellular immune response, to endogenous corticosteroid release, or to a combination of these mechanisms.

The pathogenesis of cortical petechial hemorrhages in mesenteric lymph nodes of 6 of 8 acutely infected calves is unknown. Hemorrhagic lymph nodes have been reported in spontaneous mucosal disease[13] and in experimental acute BVD viral infection.[21] Endothelial damage or defective formation or excessive activation of clotting factors and consumption of platelets are possible explanations for the bleeding tendency.

Prominence of germinal centers and expansion of paracortical areas in lymph nodes may be expected in a primary immune response to BVD viral infection.[2,4] Such a pattern was reported in lymph nodes of four bovine fetuses 120 to 165 days of gestation experimentally infected in utero with BVD virus and necropsied three weeks later.[2] Two of the fetuses produced neutralizing antibodies specific for the viral isolate used for inoculation.[4] On the basis of histologic findings and specific antibody response, the author concluded that the fetuses had made a primary immune response to BVD virus. The mesenteric lymph nodes of all four of our calves necropsied 12 days post-inoculation revealed a similar morphologic pattern and the two calves inoculated with noncytopathic TGAN virus likewise responded with specific neutralizing antibody.

Both the mild intestinal lesions of acute experimental BVD viral infection and the more severe intestinal lesions of mucosal disease are associated with patches of gut-associated lymphoid tissue (GALT). In acute experimental infection, intestinal mural edema, mucosal erosions, and petechial hemorrhages were present overlying GALT in the distal ileum, ileocecal valve, proximal colon, and distal colon. In field cases of mucosal disease, lesions reported at the same sites of intestinal lymphoid

tissue include catarrhal to fibrinous enteritis with hemorrhage and focal necrosis of Peyer's patches, catarrhal to fibrinous cecitis, colitis and proctitis with necrosis, hemorrhage and ulceration, and chronic cystic colitis 20 cm distal to the ileocecal valve.[13,33]

Demonstration of intracellular viral antigen in the GALT of 5 of 8 calves (Table 3) and isolation of BVD virus from colon of two calves (Table 1) indicates intestinal infection by BVD virus, so the lymphocytolysis and edema seen in the GALT of all eight principals are attributed to direct viral damage or to host reaction toward virus-infected cells. Because all calves except one control calf had enteric coccidiosis and all eight calves inoculated with BVD virus had intestinal mural edema, we believe that mural edema may have been induced by enteric coccidiosis[15] or by BVD virus.

In acute experimental BVD viral infection, there were very few, individual viral antigen-bearing cells in lymphoid tissues compared to mucosal disease, which is characterized by numerous individual immunoreactive macrophages and lymphocytes and by small cellular foci termed "infectious centers."[3] A possible reason is the limited number of viral replicative cycles in acute BVD viral infection contrasted with the lifelong series of replicative cycles in persistently infected cattle.

Intestinal myenteric ganglia are a likely target of bovine viral diarrhea viral infection. The central nervous system is a known site of predilection of BVD viral infection[18] and the autonomic nervous system is hypothesized to be a susceptible site. Some myenteric ganglion cells with vacuolated cytoplasm were interpreted as a degenerative change.

Ganglion cells containing BVD viral antigen detected in one calf indicates viral infection. Numerous infected and degenerated ganglion cells seen in cases of mucosal disease (C. L. Wilhelmsen, unpublished observation) may result in deranged gut motility leading to diarrhea typical of mucosal disease.

Lesions of the respiratory tract in the experimentally acutely infected calves were mild compared to respiratory tract lesions reported in field cases of mucosal disease.[13,33,34,37] In the acutely infected calves, subtle microscopic lesions were confined to the upper respiratory tract. In contrast, cattle with mucosal disease had gross and microscopic lesions reported in the upper and lower respiratory tract, including congestion of the nasal cavity, larynx and trachea, catarrhal to mucopurulent rhinotracheitis with ulcers and minor pulmonary consolidation, congestion, emphysema, and atelectasis.[13,33,34,37]

The upper respiratory tract is a predicted target of acute BVD viral infection. The tracheitis was likely induced by virus replicating in respiratory epithelium. The lack of BVD viral-induced pulmonary lesions in acute infection agrees with the finding of one group[24] which had aerosolized newborn calves with BVD virus, and contrasts with those of another group[30] which had endobronchially inoculated weanling calves with BVD virus, inducing scattered small foci of interstitial pneumonia. The viruses used here were not pneumotropic under our experimental conditions.

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LESIONS, SITES OF VIRAL REPLICATION, AND ANTIBODY RESPONSE IN SPONTANEOUS MUCOSAL DISEASE, SPONTANEOUS CHRONIC BOVINE VIRAL DIARRHEA AND INDUCED MUCOSAL DISEASE

Lesions, Sites of Viral Replication, and Antibody Response in Spontaneous Mucosal Disease, Spontaneous Chronic Bovine Viral Diarrhea and Induced Mucosal Disease

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ABSTRACT

Cattle with experimentally induced mucosal disease (n=3), spontaneous mucosal disease (n=6) and spontaneous chronic bovine viral diarrhea (n=4) were examined postmortem. Consistent findings were lymphocytic depletion of lymphoid tissues, degeneration of myenteric ganglion cells and mild adrenalitis. Intracytoplasmic viral antigen was detected in myenteric ganglia and in endocrine glandular cells. Noncytopathic virus was isolated from all cattle and cytopathic virus was isolated from 11 of 13 cattle. Of five cattle examined serologically, none completely neutralized the homologous noncytopathic virus and four of five cattle had neutralizing antibodies to their cytopathic viruses.

INTRODUCTION

Mucosal disease and chronic bovine viral diarrhea (BVD) are complex disease processes involving multiple organ systems. These diseases are differentiated by severity and duration of clinical signs. Mucosal disease is an acute disease chracterized by pyrexia, lethargy, anorexia, profuse watery diarrhea containing mucus and fresh or clotted blood, erosive lesions of oral cavity and nares, hypersalivation, mucopurulent nasal discharge, lacrimation, lameness, dehydration with acidosis and recumbency, followed by death soon after onset of clinical signs.[2,8] Chronic BVD is characterized by inappetence, progressive emaciation, rough haircoat, continual or intermittent diarrhea, chronic bloat, nasal and ocular discharge, erosive lesions of the oral cavity and lameness. Cattle with chronic BVD may survive up to 18 months, ultimately dying from severe debilitation.[2,8]

Lesions associated with mucosal disease include erosions, ulcers and cystic changes of the gastrointestinal tract; edema, hemorrhages, lymphocytic necrosis and depletion of lymphoid tissues; hemorrhages and periarteritis; catarrhal to mucopurulent rhinitis with erosions and ulcers; pulmonary emphysema; and proliferative dermatitis.[11,16,22,31,35-37,39,46] Lesions of chronic BVD include stunted growth; interdigital hyperkeratosis; interstitial pneumonia; edema, lymphocytic depletion and lack of germinal centers in lymphoid tissues; thymic atrophy; catarrhal enteritis, cystic lesions and healed erosions of the gastrointestinal tract.[12,27]

Mucosal disease and chronic BVD are sequelae of persistent infection with noncytopathic BVD virus. Persistent infection is induced during the first four months of gestation and results in persistent viremia and immune tolerance.[26] Mucosal disease, and probably chronic BVD, are induced by superinfection with cytopathic BVD virus.[11,14] However, not all combinations of noncytopathic and cytopathic virus lead to mucosal disease.[10] Further, the immune tolerance associated with persistent noncytopathic viral infection frequently does not extend to the cytopathic virus. Both neutralizing and non neutralizing antibodies are produced by persistently infected cattle after exposure to cytopathic virus.[9,10,25]

Our purpose was to compare and contrast lesions and tissue sites of viral antigen localization of experimentally induced mucosal disease, spontaneous mucosal disease and spontaneous chronic BVD in cattle with known clinical histories. Also, we wanted to determine the specificity of antibodies to noncytopathic and cytopathic viruses in serum obtained from several of those cattle immediately before death.

MATERIALS AND METHODS

Animals

Cattle (n=3) in group 1 were persistently infected with BVD-TGAN virus or BVD-VM virus (Table 1) in utero as previously described.[26] Acute mucosal disease was induced by intravenous (IV) or intranasal (IN) inoculation of 10^7 to 10^8 infectious virions of 1, 2 or 3 different isolates of cytopathic BVD viruses (Table 1). When the cattle became moribund, venous blood samples were obtained for serologic tests and the cattle were euthanatized by barbiturate overdose. Tissue samples were collected for viral isolation, histopathologic examination and immunohistochemical analysis.

Cattle (n=6) in group 2 were affected by spontaneous mucosal disease. Five of six were members of a herd of experimentally derived persistently infected cattle. The cattle were persistently infected with noncytopathic BVD-VM, BVD-7443 or BVD-TGAN viruses (Table 1). One bull (No. 5, Table 1) spontaneously developed mucosal disease while at the National Animal Disease Center (NADC), but was not in the herd of persistently infected cattle. This bull was identified retrospectively as persistently infected by viral isolation from stored serum samples obtained over a one year period. All 6 cattle were euthanatized in extremis or died naturally. Venous blood samples for serologic tests were obtained prior to euthanasia.

Cattle (n=4) in group 3 developed spontaneous chronic BVD. These cattle were members of a herd of cattle with persistent infection. The cattle were persistently infected with BVD-WVA virus or were purchased

Table 1. Experimental cattle and viruses

Group number	Bovine number	Sex	Noncytopathic virus	Cytopathic virus inoculated	Age at death	Cytopathic virus isolated
1	1	M	TGAN	TGAC	6 months	Yes
1	2	M	TGAN	TGAC SNC 2110	18 months	Yes
1	3	M	VM	TGAC Singer	9 months	Yes
2	4	F	VM	None	2 years	Yes
2	5	M	1185	None	2 years	Yes
2	6	F	7443	None	2 years	Yes
2	7	F	7443	None	2 years	Yes
2	8	F	7443	None	2 years	Yes
2	9	F	TGAN	None	2 years	Yes
3	10	F	NEB	None[b]	5 years	Yes
3	11	MC[a]	NEB	NADL[b]	8 years	Yes
3	12	MC	9789	None	3 years	Yes
3	13	F	WVA	None	6 years	No
4	14	F	VM	None	18 months	No
4	15	F	NEB	None	6 years	No

[a]MC = male, castrated.
[b]NADL = porcine-adapted NADL modified live virus vaccine inoculated at 4 years of age.

naturally persistently infected with BVD-NEB virus (Table 1) and maintained at NADC for at least four years before developing clinical chronic BVD. Cattle died or were euthanatized in extremis.

Cattle (n=2) in group 4 served as controls and were persistently infected with noncytopathic BVD-VM or BVD-NEB. These cattle did not show clinical signs of mucosal disease or chronic BVD. One died naturally and the other was euthanatized because of a debilitating neurologic condition. Cattle in groups 2-4 were maintained in outdoor drylots or on pasture with adjoining three-sided sheds.

Viruses and Viral Isolation

Cytopathic viruses used to induce mucosal disease were purified by three successive plaque purifications. The purified viruses were then passed at least three times at a multiplicity of infection of 1 to 10 to ensure stability of cytopathic effect. Viral isolation was attempted from all cattle by inoculation of bovine turbinate (BT) cell culture monolayers with serum or 20% suspensions of spleen and colon or esophagus followed by incubation at 37°C for seven days. Monolayers were observed daily for cytopathic effect (CPE).[21] Cytopathic viruses were then purified by the aforementioned procedures. Noncytopathic viruses were purified by three successive passages at terminal dilution.

Several noncytopathic viruses (7443, VM, TGAN, WVA; Table 1) and cytopathic viruses (TGAC, Singer; Table 1) used in this study were previously isolated from cattle in the United States.[11,26] Other noncytopathic viruses (1185, NEB, 9789) were isolated from cattle in the present study

(Table 1). Cytopathic BVD-SNC and BVD-2110 viruses were isolated, respectively, from cattle Nos. 9 and 10 (Table 1).

Viral Neutralization

Stocks of noncytopathic and cytopathic viruses were grown and titered using standard methods.[15] Partially purified IgG was prepared from serum samples by ammonium sulfate precipitation followed by gel filtration chromatography.[10] The partially purified IgG was free of noncytopathic BVD virus, as determined by virus isolation and direct fluorescent antibody (FA) procedure.[9] Immunoglobulin was prepared from serum of a control cow hyperimmunized against BVD-NADL virus. Immunoglobulin from each animal was concentrated to 23 mg protein/ml. Viral neutralization tests with cytopathic viruses were done by standard methods.[38] Viral neutralization tests with noncytopathic viruses were done as previously described.[12] All cell culture reagents used for virological techniques were free of adventitious BVD virus and antibody to BVD virus.

Radioimmunoprecipitation (RIP)

This was done as previously described.[9] Briefly, monolayers of BT cells were infected with noncytopathic or cytopathic viruses at a multiplicity of infection of greater than 10. After 16 hours incubation, intracellular viral proteins were radiolabelled with 200 microcuries of [^{35}S] L-methionine and L-cystine in methionine deficient MEM. After 24 to 48 hours, radiolabelled virus-infected cells were lysed. Infected BT cell lysate absorbed with immunoabsorbent (Protein A Sepharose CL-4B, Pharmacia Fine Chemicals, Piscataway, NJ) was incubated with IgG preabsorbed with noninfected BT cell lysate. Immunosorbent freshly absorbed

with noninfected BT cell lysate was incubated with the mixture of infected BT cell lysate and immunoglobulin. The RIPs were washed, suspended in electrophoresis sample buffer, heated, and analyzed by SDS-polyacrylamide gel electrophoresis.[23] Resolving gels were 10% or 12.5% acrylamide and electrophoresis was done at a constant power of 12 watts per gel. Fixed and dried gels were exposed to film (Kodak SB Medical X-ray Film, Eastman Kodak Co., Oak Brook, IL) at -90°C for 120 hours.

Histopathology

Tissues collected were adrenal gland, abomasum, brain, cecum, distal colon, esophagus, eye, gall bladder, gonad, heart, ileocecal valve, jejunum, kidney, liver, lung, mediastinal and mesenteric lymph nodes, nasal mucosa, pancreas, pituitary gland, proximal colon, rumen, salivary gland, spleen, thyroid, thymus, trachea, and urinary bladder. Tissues for histopathology were fixed in 10% neutral buffered formalin, processed by standard techniques and stained with hematoxylin and eosin (HE).

Immunohistochemistry

Tissues collected were adrenal gland, abomasum, brain, epidermis, esophagus, gall bladder, ileum, lacrimal gland, lung, lymph node, nasal mucosa, pancreas, pituitary gland, proximal colon, rumen, salivary gland, and thyroid gland. Tissues were frozen unfixed at -80°C in 2.5% methyl cellulose or in commercial embedding medium for frozen tissue specimens (O.C.T. Compound, Miles Laboratories, Naperville IL). All tissues were reacted with two or more monoclonal antibodies (MABs) of known specificity for BVD virus.[12] The MAB's were in serum-free Dulbecco's minimum essential medium supplemented with insulin and transferrin. Cryosections on

poly-L-lysine coated glass microslides were air dried and fixed in 100% chilled acetone (-20°C). Rehydration was done with Tris buffer,[40] which was also used for all dilutions and washes. After blocking endogenous peroxidase activity,[24] nonspecific reactivity was blocked with 0.5% horse serum in Tris buffer at 22°C for 20 minutes. Tissue sections were coated with a 1:10 dilution of MAB in Tris buffer and incubated for 16 hours at 4°C in a humidified chamber. Immunoperoxidase staining was done with biotinylated equine antimouse IgG and streptavidin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA), followed by application of the substrate (equal volumes of 0.1% diaminobenzidine tetrahydrochloride in 0.1M Tris buffer pH 7.2 and 0.03% hydrogen peroxide). Tissue sections were postfixed in formol saline, counterstained with hematoxylin, dehydrated and mounted. Controls comprised known negative samples from two calves free of BVD virus and having antiBVD viral antibody titers of 1:4 or less, replacement of MAB by serum supplemented MEM and substitution of specific MABs with nonspecific MABs.

RESULTS

Group 1

One calf became anorectic and developed diarrhea with blood flecks 13 days after initial exposure to cytopathic virus. Mucosal disease was not induced in two calves by initial exposure to cytopathic virus. One of these two calves developed bloody diarrhea four and a half months after inoculation with the second cytopathic virus (Table 1). In the other calf, transient lacrimation, partial anorexia and soft feces occurred between 8 and 24 days after inoculation with the initial cytopathic virus, but signs resolved spontaneously. Severe mucosal disease with watery diarrhea and mucopurulent nasal discharge developed in this calf two months after exposure to the third cytopathic virus.

Gross (Table 2) and microscopic lesions of the gastrointestinal and lymphoid systems were similar in all calves. The proximal colon 25 cm distal to the ileocecal valve had mucosal erosions and mural edema (Fig. 1). There was eosinophilic enterocolitis with crypt abscesses, prolapsed crypts and cystic glands. Myenteric ganglion cells in all calves had peripheral cytoplasmic vacuolation (Fig. 2). There was upper alimentary tract mucosal cell vacuolation, necrosis and detachment. Lymphoid tissues had marked follicular lymphocytic depletion or necrosis or a few small indistinct follicles lacking germinal centers.

Consistent lesions of other tissues were multiple lymphoplasmacytic and eosinophilic foci in renal and adrenal cortices, portal triads and gall bladder submucosa. Intracytoplasmic BVD viral antigen was

Table 2. Gross lesions of gastrointestinal and lymphoid systems

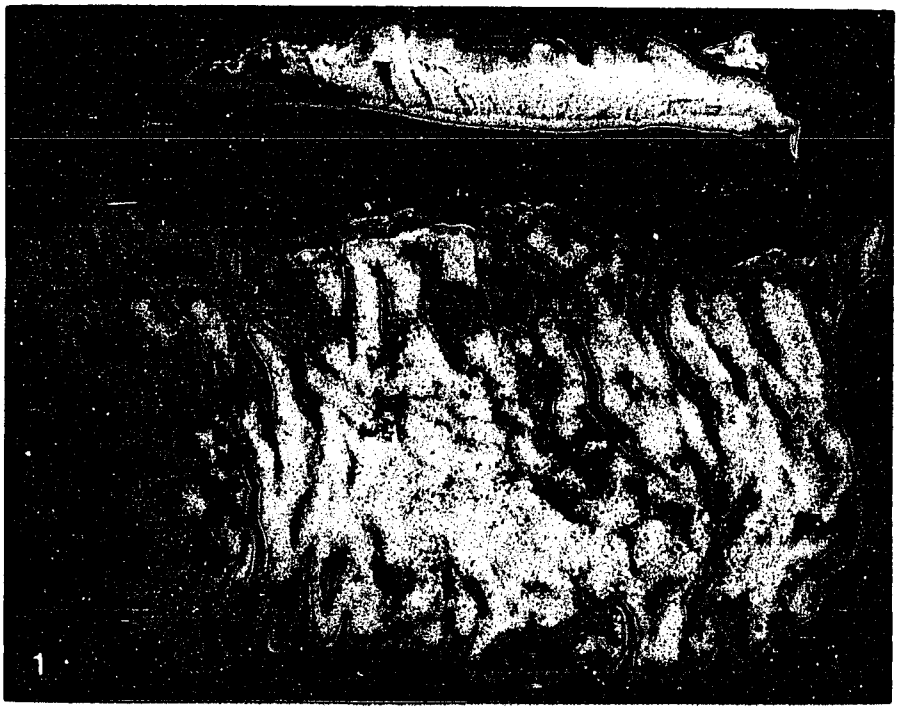
Group number	1	1	1	2	2	2	2	2	2	3	3	3	3	4	4
Bovine number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Esophageal erosions	–[a]	–	–	+[b]	+	–	+	–	–	–	–	–	+	–	–
Abomasal ulcers	–	+	+	+	+	–	+	–	–	–	+	+	+	–	–
Thinning of Peyer's patches	+	+	+	–	+	+	+	+	+	–	–	+	+	–	–
Edema of proximal colon	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–
Rectal hemorrhages	–	+	+	+	–	–	+	–	–	+	–	–	+	–	–
Edema and erosions of gall bladder	–	+	–	–	+	–	–	–	–	–	+	+	–	–	+
Lymph node hemmorhages	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–
Lymph node atrophy	–	+	+	–	–	–	+	–	–	–	+	–	+	–	–
Proliferative lymph nodes	+	–	–	+	+	–	–	–	–	+	–	+	–	–	–
Proliferative hemal nodes	+	–	–	–	–	+	+	+	+	–	–	+	–	+	–

[a] – = Lesion absent.

[b] + = Lesion present.

Figure 1. Proximal colon, calf with induced mucosal disease (No. 3). Mucosal petechial hemorrhages and severe edema of the smooth muscle layer

Figure 2. Proximal colon, calf with induced mucosal disease (No. 1). Peripheral cytoplasmic vacuolation of myenteric ganglion cells. HE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consistently found in myenteric ganglion cells, in crypt cells and in mononuclear cells of gut associated lymphoid tissue (GALT) and of mesenteric lymph nodes.

Noncytopathic and cytopathic virus was isolated from spleen of all three cattle at death (Table 1). Complete neutralization of noncytopathic virus was not detected in any calf at death. Two calves (Nos. 1 and 2) had partial neutralizing activity to the noncytopathic virus. Complete neutralization of one or more cytopathic viruses was detected in all calves at death (Table 3). Calf No. 3 had partial neutralizing activity for the second inoculated cytopathic virus and the cytopathic virus isolated postmortem. All calves produced antibodies that precipitated several viral induced proteins of the calves' heterologous cytopathic viruses (Figs. 9-11). Two calves (Nos. 2 and 3) had precipitating antibodies that detected the homologous noncytopathic virus (Figs. 10 and 11).

Group 2

All cattle had diarrhea with blood flecks for one day to one month before euthanasia or death, except No. 9 (Table 1) which was observed to have only tenesmus. Dyspnea and mucous nasal discharge were observed in 5 of 6 cattle. Three cattle that shared a pen (Nos. 6,7,8; Table 1) were 2-year-old heifers all persistently infected with BVD-7443 virus. One heifer (No. 6) developed mucosal disease and died. The other 2 heifers appeared healthy at the time of the first heifer's death, but developed mucosal disease and died within 15 to 20 days. Two other cattle were penned together (Nos. 4 and 13; Table 1). The cow (No. 13) had intermit-

Table 3. Results of viral neutralization and radioimmunoprecipitates

Group number	Bovine number	Virus	Viral neutralization	Antibody to 56 KD protein
1	1	TGAN	-[a]	-[b]
		TGAC	+[c]	+[d]
1	2	TGAN	-	-
		TGAC	+	+
		SNC	+	-
		2110	-	-
		CPV[e]	-	-
1	3	VM	-	-
		TGAC	+	+
		Singer	-	+
		CPV	-	-
2	4	VM	-	-
		CPV	-	+
3	11	NEB	-	-
		NADL	+	-
		CPV	-	-

[a]- = Absence of complete viral neutralization.

[b]- = Antibody to 56 KD protein not detected.

[c]+ = Presence of complete viral neutralization.

[d]+ = Antibody to 56 KD protein detected.

[e]CPV = Cytopathic virus isolated at death.

Figure 9. Radioimmunoprecipitations of noncytopathic BVD-TGAN and cytopathic BVD-TGAC viral proteins labelled with [^{35}S] L-methionine and cystine and reacted with immunoglobulin from a calf with induced mucosal disease (No. 1). Lane 1 contains ^{14}C-methylated molecular weight standards, with the molecular weights in kilodaltons on the left. Lanes 2 and 3 contain the results of reacting hyperimmune globulin raised against cytopathic BVD-NADL virus with viral proteins of cytopathic BVD-TGAC (lane 2) and noncytopathic BVD-TGAN (lane 3). Lanes 4 and 5 contain the results of reacting IgG from a calf with induced mucosal disease (No. 1) before receiving cytopathic BVD-TGAC with viral proteins of cytopathic BVD-TGAC (lane 4) and noncytopathic BVD-TGAN (lane 5). Lanes 6 and 7 contain the results of reacting IgG from calf No. 1 16 days after receiving cytopathic BVD-TGAC virus with the viral proteins of BVD-TGAC (lane 6) and BVD-TGAN (lane 7). Lane 8 contains the results of reacting hyperimmune anti-BVD-NADL antiglobulin with uninfected cell proteins. The approximate molecular weights in kilodaltons of the viral induced proteins are on the right. Cytopathic virus has an extra viral protein of 80 kilodaltons not found in noncytopathic virus

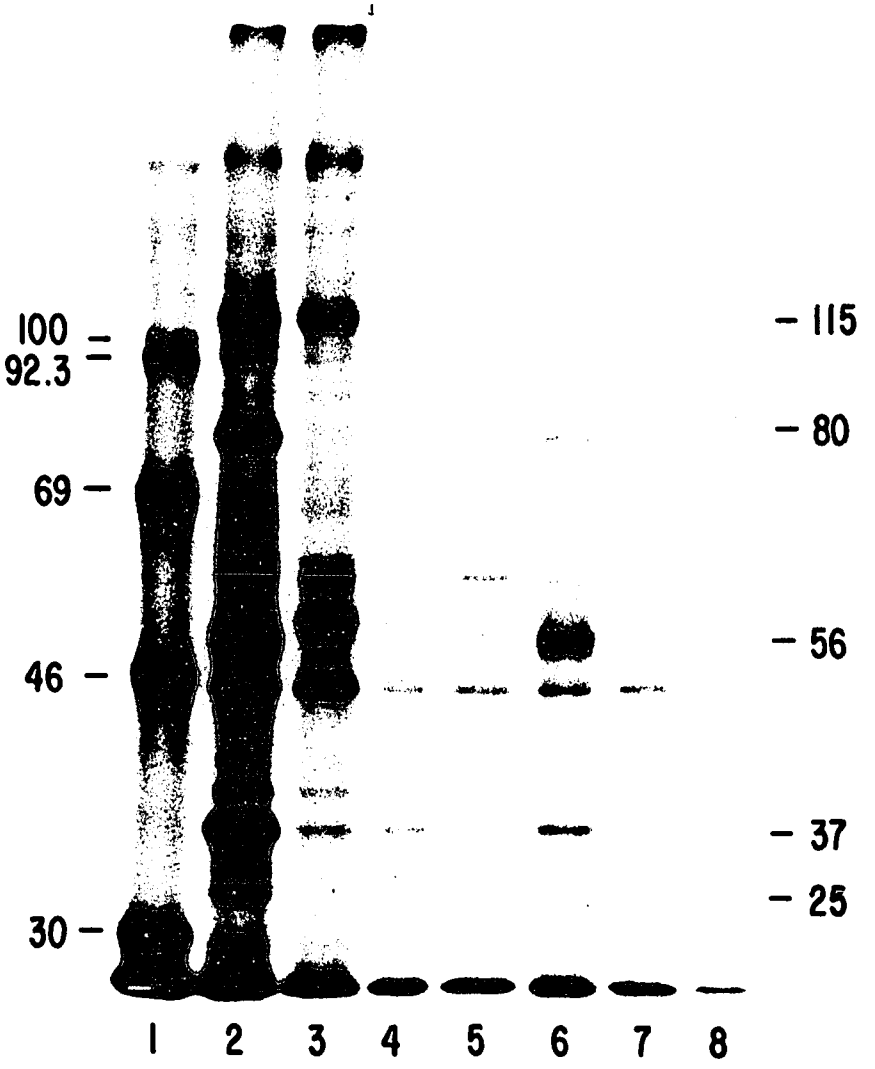
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Figure 10. Radioimmunoprecipitations of viral proteins of non-cytopathic BVD-TGAN virus and 4 cytopathic viruses (3 inoculated and one isolated) labelled with [^{35}S] L-methionine and cystine and reacted with immunoglobulin from a calf with induced mucosal disease (No. 2). Lane 1 contains ^{14}C-methylated molecular weight standards, with the molecular weights in kilodaltons to the left. Lanes 2-6 contain the results of reacting hyperimmune globulin raised against BVD-NADL virus with viral proteins of noncytopathic BVD-TGAN (lane 2), cytopathic BVD-TGAC (lane 3), cytopathic BVD-SNC (lane 4), cytopathic BVD-2110 (lane 5), and the cytopathic virus isolated postmortem from a calf with induced mucosal disease (No. 2; lane 6). Lanes 7-11 contain the results of reacting No. 2's immunoglobulin with labelled viral proteins of BVD-TGAN (lane 7), BVD-TGAC (lane 8), BVD-SNC (lane 9), BVD-2110 (lane 10), and the cytopathic virus from No. 2 (lane 11). The approximate molecular weights of viral induced proteins in kilodaltons are indicated on the right. The 80 kilodalton protein of BVD-2110 is visible on longer exposures

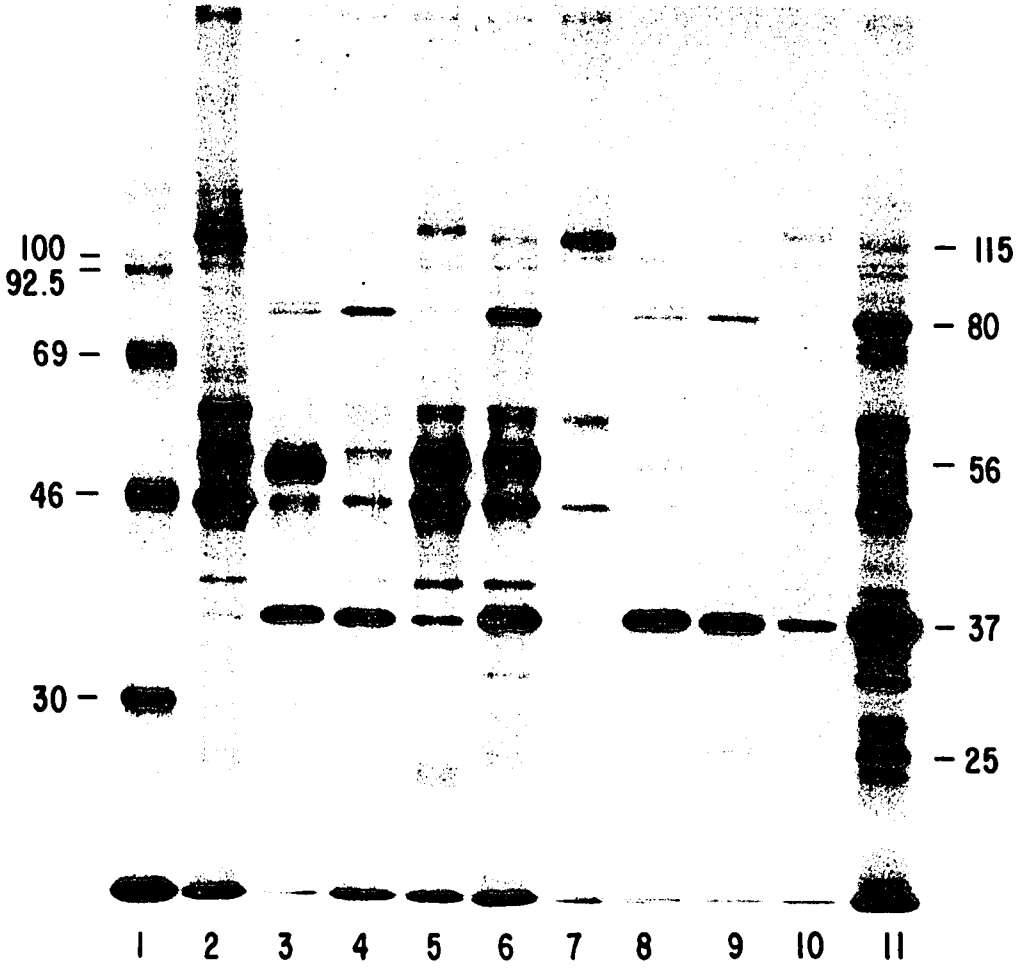
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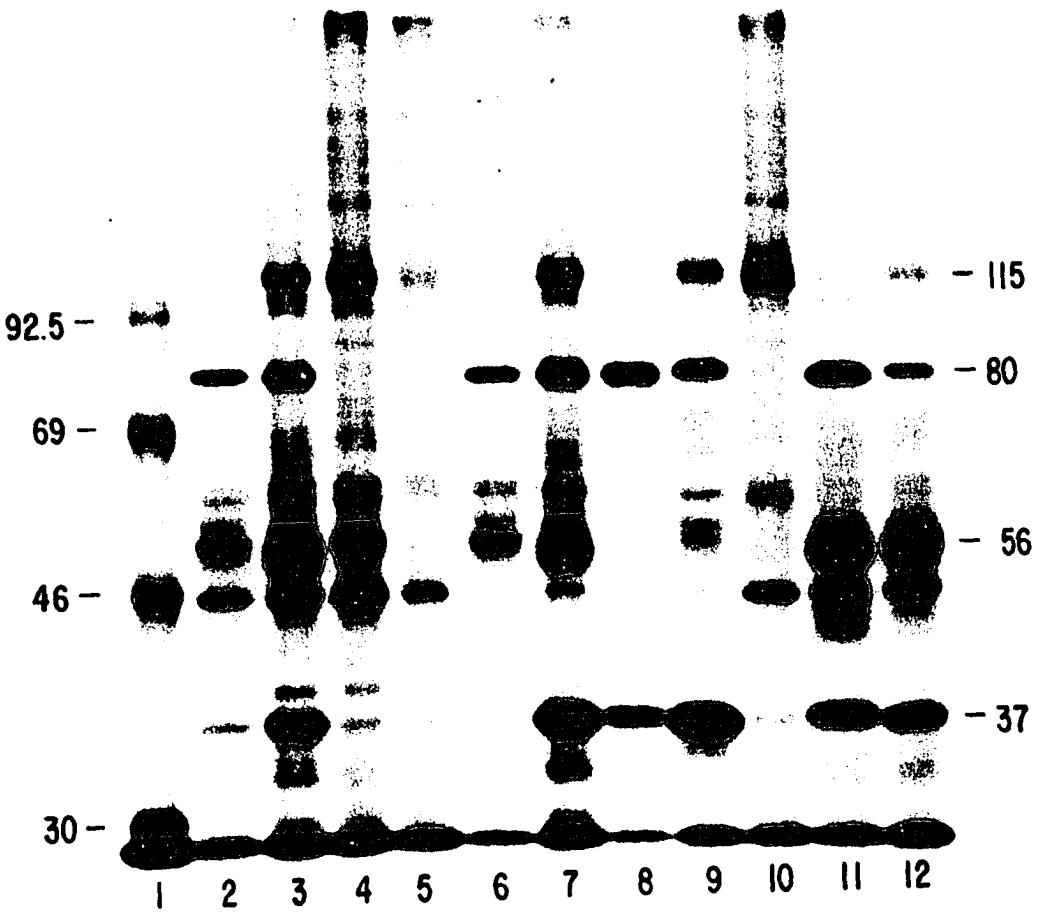
Figure 11. Radioimmunoprecipitations of noncytopathic and cytopathic viral proteins labelled with [^{35}S] L-methionine and cystine and reacted with immunoglobulin from cattle with induced and spontaneous mcosal disease. Lane 1 contains ^{14}C-methylated molecular weight standards, with the molecular weights in kilodaltons to the left. Lanes 2-4 contain the results of reacting hyperimmune globulin raised against BVD-NADL virus with labelled viral proteins of cytopathic BVD-Singer (lane 2) and BVD-TGAC (lane 3) and noncytopathic BVD-VM (lane 4). Lanes 5-8 contain the results of reacting IgG from a calf with induced mucosal disease (No. 3) with labelled viral proteins of BVD-VM (lane 5), BVD-Singer (lane 6), and BVD-TGAC (lane 7) and the cytopathic virus isolated from No. 3 at death (lane 8). Lanes 9 and 10 contain the results of reacting IgG from a heifer with spontaneous mucosal disease (No. 4) with labelled viral proteins of the cytopathic virus isolated from No. 4's tissues (lane 9) and noncytopathic BVD-VM (lane 10). Lanes 11 and 12 contain the results of reacting hyperimmune globulin raised against BVD-NADL virus with labelled proteins of No. 3's cytopathic virus (lane 11) and No. 4's cytopathic virus (lane 12). The approximate molecular weights in kilodaltons of viral induced proteins are indicated to the right of lane 12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tent diarrhea for six months. Three months after the cow became ill, her healthy penmate (No. 4) suddenly succumbed to fatal mucosal disease.

Gross (Table 2) and microscopic lesions and tissue viral antigen distribution of cattle with spontaneous mucosal disease were the same as cattle with induced mucosal disease. Noncytopathic and cytopathic viruses were isolated from spleen or intestine or esophagus of all cattle (Table 1), but purification of the noncytopathic and cytopathic viruses isolated from only one heifer with spontaneous mucosal disease (No. 4; Table 1) was possible. This heifer lacked detectable neutralizing antibodies to the homologous noncytopathic virus. The cytopathic virus isolated from tissues at postmortem was not completely neutralized (Table 3), although partial neutralizing activity was detected. Antibody from this heifer precipitated viral induced proteins of her cytopathic and noncytopathic viruses (Fig. 11).

Group 3

Cattle had diverse clinical signs including anorexia, weight loss, intermittent diarrhea, constipation, mucopurulent nasal discharge, dyspnea, cough and subcutaneous edema of the legs. Consistent gross lesions (Tables 2 and 4) were erosions and ulcers of the upper alimentary tract, and pulmonary emphysema. Three cattle had proximal colonic mural edema. One cow lacked mural edema, but had proximal colonic multifocal mucosal nodules with red-black margins (Fig. 3). Other gross lesions are listed in Table 4.

Consistent microscopic lesions were peripheral vacuolation of myenteric ganglion cells; diffuse severe lymphocytic depletion of GALT; small

Table 4. Additional gross lesions in cattle with chronic BVD

	10	11	12	13
Flaccid cecum	+[a]	+	-[b]	-
Pitted kidneys	-	+	-	+
Pulmonary emphysema	+	+	+	+
Pulmonary consolidation	+	-	-	+
Tracheal exudate	+	-	-	+
Cerebral atrophy	-	+	-	+

[a] + = Lesion present.

[b] - = Lesion absent.

Figure 3. Proximal colon, cow with chronic BVD (No. 13). Multifocal mucosal nodules

Figure 4. Proximal colon, cow with chronic BVD (No. 13), HE. Submucosal fibrosis and submucosal glands devoid of periglandular lymphoid tissue

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lymphoid follicles lacking germinal centers in mesenteric lymph nodes; renal and adrenal multifocal lymphoplasmacytic infiltrates; and pulmonary alveolar and interlobular emphysema. Three cattle had cystic colonic submucosal glands. One cow had proximal colonic mild mucosal goblet cell hyperplasia and moderate submucosal fibrosis forming mucosa-covered nodules that projected into the intestinal lumen (Fig. 4). One steer had focal neutrophilic-lymphocytic ganglioneuritis. Additional findings were renal leptospirosis, generalized amyloidosis, granulomatous abdominal fat necrosis, bronchitis, bronchopneumonia and pulmonary abscesses. Intracytoplasmic BVD viral antigen was detected in myenteric ganglion cells (Fig. 5), intestinal crypt cells and mononuclear cells of GALT and lymph nodes.

Noncytopathic virus was isolated from all cattle after death. Cytopathic virus was isolated from 3 of 4 cattle (Table 1). Neutralizing antibodies to noncytopathic virus were not detected in IgG from a steer with chronic BVD (No. 11; Table 1). Number 11's IgG completely neutralized cytopathic BVD-NADL (Table 3) and partially neutralized the cytopathic virus isolated postmortem. Antibodies were detected to proteins of BVD-NADL virus, but not to No. 11's noncytopathic and cytopathic viruses (data not shown).

Group 4

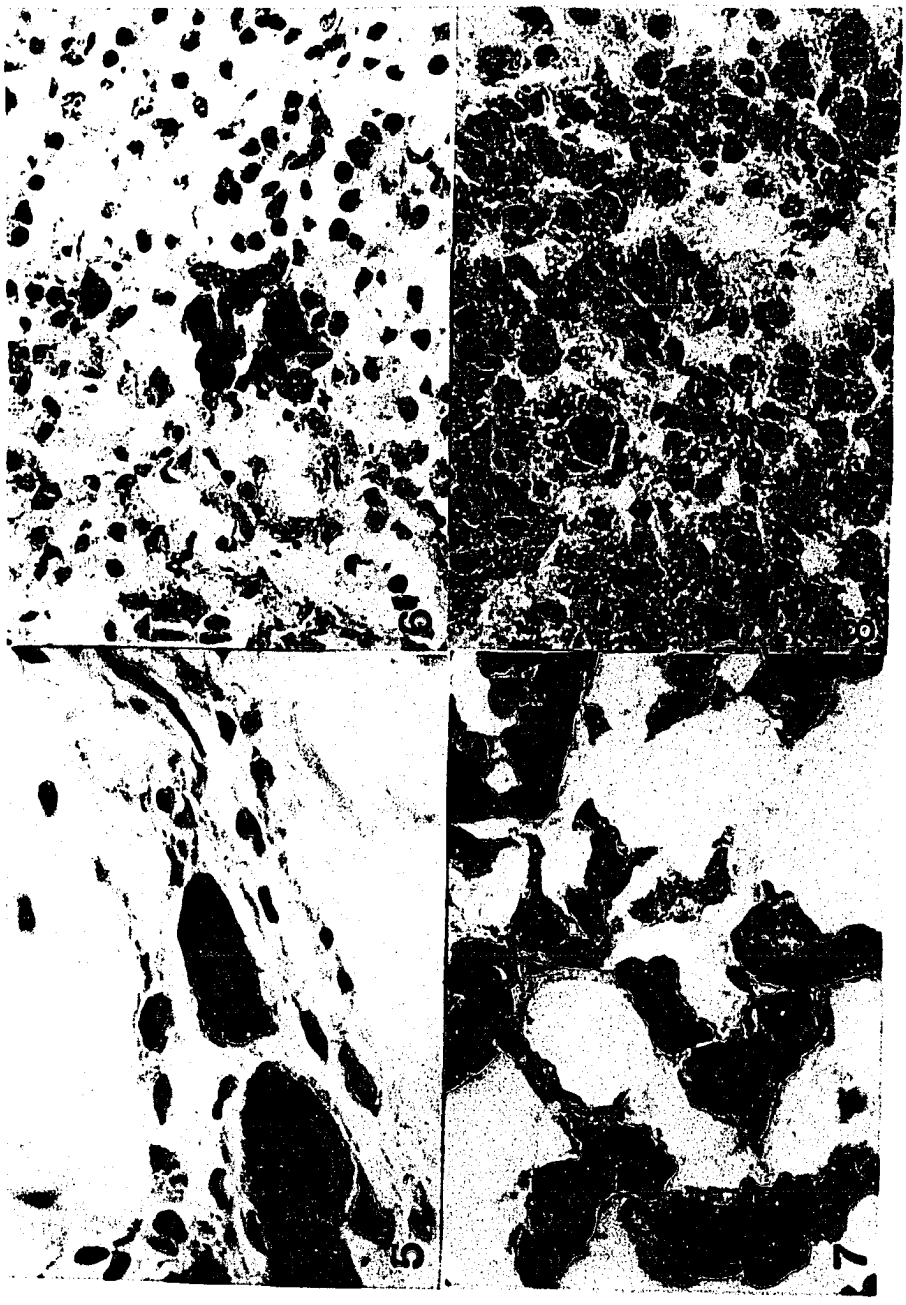
One persistently infected female (No. 14, Table 1) had ataxia and head tilt which progressed to paralysis. The other female (No. 15, Table 1) died suddenly, without showing clinical signs. There were no consistent gross lesions. Consistent microscopic lesions were mild peripheral vacuo-

Figure 5. Proximal colon, bull with spontaneous mucosal disease (No. 5). Immunoperoxidase staining of intracytoplasmic BVD viral antigen in a myenteric ganglion cell

Figure 6. Pancreas, steer with chronic BVD (No. 11). Immunoperoxidase staining of intracytoplasmic BVD viral antigen in acinar cells

Figure 7. Adenohypophysis, cow with chronic BVD (No. 13). Immunoperoxidase staining of intracytoplasmic BVD viral antigen in pituitary cells. The pale amorphous extracellular material is amyloid

Figure 8. Adrenal gland, steer with chronic BVD (No. 11). Immunoperoxidase staining of intracytoplasmic BVD viral antigen in adrenocortical cells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lation of myenteric ganglion cells and renal and adrenal lymphocytic foci. Other findings in No. 14 were necrotizing arteritis, lymphocytic perivascular cuffs and multiple infarcts in the brain and lymphoid hyperplasia in hemal nodes. Other lesions in No. 15 were pulmonary emphysema, bronchiolitis and interstitial pneumonia with intracellular bovine respiratory syncytial viral antigen. Intracellular BVD viral antigen was detected in lymph node mononuclear cells and myenteric ganglion cells. Noncytopathic virus was isolated from the spleen of both cattle.

Additional Findings

Intracytoplasmic viral antigen was detected in adrenocortical cells (Fig. 8) and cerebral neurons of cattle with chronic BVD and persistent infection. Other endocrine organs of cattle with chronic BVD with intracytoplasmic viral antigen were adenohypophysis (Fig. 7), thyroid gland and pancreatic islets. Viral antigen was also in esophageal mucosal cells of cattle with spontaneous mucosal disease, chronic BVD and persistent infection. In cattle with chronic BVD, mucosal cells of nares, rumen, abomasum and gall bladder and acinar and ductal cells of salivary, lacrimal and nasal submucosal glands and exocrine pancreas (Fig. 6) contained intracytoplasmic viral antigen.

DISCUSSION

In this study, spontaneous chronic BVD was differentiated clinically from spontaneous mucosal disease as follows: (1) weight loss, (2) debilitation, and (3) clinical signs referable to the gastrointestinal tract with a duration exceeding one month. Cattle with spontaneous chronic BVD had several enteric and lymphoid lesions reminiscent of mucosal disease, but lesions were less severe and chronic. Finding lesions suggestive of mucosal disease (alimentary tract erosions, cystic intestinal submucosal glands, lymphocytic depletion of GALT) aided differentiation of cattle with chronic BVD from persistently infected "poor doers". Several lesions in other systems not typical of mucosal disease were attributed to secondary bacterial infection or intercurrent disease. The severe lymphocytic depletion of lymphoid tissues of cattle with chronic BVD may result in reduced immune responsiveness and increased susceptibility to secondary infections.

The most consistent enteric lesion, present in all four groups, was peripheral cytoplasmic vacuolation of myenteric ganglion cells, interpreted as a degenerative change. Bovine viral diarrhea viral antigen was detected within intestinal ganglion cells in cattle in all groups. Mild focal colonic myenteric ganglioneuritis, as seen in a steer with chronic BVD, may be a host cellular response to infected ganglion cells. A similar infiltration around colonic intramural ganglia was described in induced mucosal disease.[32] Viral infection of ganglion cells may lead to disturbed autonomic neural function resulting in deranged gut tone and motility.

A consistent finding in all four groups was mild multifocal adrenocortical lymphoplasmacytic and eosinophilic cellular infiltrates, previously described in field cases of mucosal disease.[47] Viral antigen was detected in adrenocortical cells in cattle with chronic BVD and persistent infection. Hence, it is likely the mild adrenalitis found in several cattle was a host cellular response attributed to adrenal infection. Adrenal glands of persistently infected cattle are known to be infected by virus[3] and to contain viral antigen.[26]

Other endocrine organs with intracellular BVD viral antigen were pituitary gland, thyroid gland and pancreatic islets. In contrast to one study,[20] but in accord with another study,[4] BVD viral antigen was detected within glandular cells of the adenohypophysis. Pestiviral antigen has also been detected in pituitary glandular cells of lambs congenitally infected with border disease virus.[1] BVD viral antigen within thyroid follicular and pancreatic cells has been reported previously in a persistently infected bull.[4] Lambs with border disease are also known to have viral antigen within pancreatic cells.[45]

The most common reservoirs for persistent viruses are the differentiated cells of the nervous and immune systems,[41] but other favored sites for viral persistence are the endocrine system[41] and basal cells on epithelial surfaces.[28] Our immunohistochemical study using monoclonal antibodies to detect sites of BVD viral antigen localization in tissues of persistently infected cattle with induced or spontaneous mucosal disease or chronic BVD showed that nervous, lymphoid, endocrine and epithelial tissues were locations of BVD viral antigen. We confirmed and extended the findings of others who used bovine, caprine, ovine and

porcine polyclonal sera to localize BVD viral antigen in field cases of persistent infection and mucosal disease.[4,5-7,17,20,27,33,44]

All cattle with induced and spontaneous mucosal disease developed diarrhea and dehydration, resulting in death or euthanasia in extremis. Death was likely due to poor compensatory response to shifts in acid-base status and to disturbances in fluid and electrolyte balance. In contrast to the acute disease in these cattle, cattle with chronic BVD developed a chronic wasting disease with anorexia and weight loss resulting in progressive debilitation and increased susceptibility to infection by other pathogens. The cause of death in one cow with chronic BVD that died naturally was not established with certainty because of concurrent involvement by several disease processes (leptospirosis, amyloidosis, pneumonia).

The pathogenesis of progressive debilitation in cattle with chronic BVD is unclear. Persistent viral infection of endocrine cells hypothetically could interfere with specialized cellular function resulting in altered levels of hormone synthesis and a change in homeostasis.[30] Altered growth hormone synthesis by infected pituitary cells could lead to growth disturbances such as stunting of persistently infected cattle.[18,25,34,43] Altered thyroglobulin secretion by infected thyroid follicular cells could adversely affect the basal metabolic rate. In lambs congenitally infected with border disease virus, thyroid follicular cells contain border disease viral antigen and serum thyroid hormone levels of affected lambs are decreased.[1] Infection of pituitary or adrenocortical cells could result in indirect or direct interference with adrenocorticoid synthesis. Decreased adrenocorticoid levels could lower a

persistently infected cow's ability to cope with the stress of secondary infection.

The source of cytopathic virus in cattle with spontaneous mucosal disease and chronic BVD remains undetermined. Two possible sources are mutation from the endogenous persistent noncytopathic virus and transmission from other cattle infected with cytopathic virus. Both mechanisms probably occur. The two cattle (Nos. 7 and 8) penned with No. 6 probably were infected by contact with No. 6 after No. 6 developed mucosal disease. Number 6's cytopathic virus may have arisen by mutation in biotype of No. 6's persistent noncytopathic virus. The evidence for transmission of the cytopathic virus from No. 6 to Nos. 7 and 8 is circumstantial, but is supported by the onset of clinical signs in Nos. 7 and 8 two weeks after the death of No. 6. Likewise, the cytopathic virus isolated from a heifer with mucosal disease (No. 4) may have arisen by mutation in her penmate, a cow with chronic BVD (No. 13), followed by transmission to No. 4.

We found that cattle with spontaneous mucosal disease and chronic BVD produced antibodies that neutralized their cytopathic viruses and precipitated cytopathic viral induced proteins. Our results differ from those reported in a serologic field study of mucosal disease.[19] The different methods used in the cited study and our study may explain the disparate findings. Cattle sera were tested for antibodies to only one cytopathic virus (BVD-Singer) in the cited study, whereas we used individual animals' own cytopathic viruses in our serologic tests. Our cattle usually detected 115 and 80 kilodalton viral induced proteins of their cytopathic

viruses. Less often, the 53 to 56 kilodalton protein was recognized. We saw a consistent association between precipitation of the 53 to 56 kilodalton viral induced protein and complete viral neutralization of one cytopathic virus (BVD-TGAC). A correlation between viral neutralization and detection of the 53 kilodalton protein has been reported.[9]

Results of viral neutralization and precipitation tests with several other viruses did not correspond as closely. Antibodies of two cattle completely neutralized inoculated cytopathic viruses without precipitating the 53 to 56 kilodalton viral-induced protein. A lower serum dilution was used in viral neutralization (1:2) than in RIP (1:6). Hence, viral neutralization was likely more sensitive than RIP at detecting low antibody levels. Antibodies of two other cattle precipitated the 53 to 56 kilodalton viral-induced protein of two cytopathic viruses without completely neutralizing the viruses. (Partial viral neutralizing activity was detected.) The precipitating antibodies may have been directed to non-neutralizing or poorly neutralizing epitopes on the 53 to 56 kilodalton protein.[9] Sera of two calves had partial neutralizing activity for the homologous noncytopathic virus (BVD-TGAN) without detectable precipitation of the 53 to 56 kilodalton protein. The calves' sera either had low levels of neutralizing antibody or had nonspecific toxic substances with antiviral activity. The second hypothesis is less likely because the sera were extensively dialyzed to reduce the risk of nonspecific toxicity.

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GENERAL SUMMARY AND DISCUSSION

The first study established that experimental infection of calves with either biotype of bovine viral diarrhea (BVD) virus induced similar mild lesions of the enteric, lymphoid and respiratory systems. Consistent gross lesions were proximal colonic mural edema and mesenteric lymph nodal hemorrhages. Consistent microscopic lesions were acute mild enterocolitis with peripheral vacuolation of myenteric ganglion cells; mild follicular lymphocytic depletion of gut-associated lymphoid tissue (GALT) and mesenteric lymph nodes; and acute mild tracheitis.

The second study showed that cattle with experimental and spontaneous mucosal disease had similar generally severe lesions of the gastrointestinal and lymphoid systems. Consistent gross lesions of induced and spontaneous mucosal disease were colonic mural edema and thinning of Peyer's patches. Lymph nodal petechiation was uniformly present only in induced mucosal disease. Consistent microscopic lesions of induced and spontaneous mucosal disease were acute moderate enterocolitis with peripheral vacuolation of myenteric ganglion cells; moderate to severe follicular lymphocytic depletion of GALT and mesenteric lymph nodes; multifocal mucosal necrosis and inflammation of variable severity in the upper alimentary tract; and mild lymphocytic adrenalitis.

The second study also showed that cattle with chronic BVD had mild lesions, several suggestive of mucosal disease. The only consistent gross lesion was pulmonary emphysema. Consistent microscopic lesions were peripheral vacuolation of myenteric ganglion cells; severe diffuse lymphocytic depletion of GALT; small lymphoid follicles without

germinal centers in mesenteric lymph nodes; mild multifocal necrosis of upper alimentary tract mucosa; alveolar and interlobular emphysema; and mild lymphocytic adrenalitis.

Compared to cattle with mucosal disease and chronic BVD, persistently infected cattle had few consistent lesions. There were no consistent gross lesions. Consistent microscopic changes were mild peripheral vacuolation of myenteric ganglion cells and mild lymphocytic adrenalitis.

Immumocytochemical results indicated that BVD virus is lymphotropic, neurotropic and epitheliotropic. In both studies, intracellular BVD viral antigen was detected in lymphoid tissue mononuclear cells (presumably lymphocytes) and autonomic ganglia of infected cattle. Viral antigen was also found in diverse endocrine and exocrine epithelial cells and in cerebral neurons of cattle in the second study.

Both studies showed that cattle infected with BVD virus produced specific antiviral antibodies. In the first study, calves infected with noncytopathic virus produced neutralizing antibody to their noncytopathic virus. In the second study, cattle with induced and spontaneous mucosal disease produced neutralizing and precipitating antibodies to their cytopathic viruses. However, the onset of induced mucosal disease could not be correlated with detection of antibodies to the inoculated cytopathic virus. Three calves with induced disease were inoculated with the same cytopathic virus (BVD-TGAC). All calves produced antibodies that neutralized BVD-TGAC virus and precipitated the 56 kilodalton viral induced protein of this virus. Yet, only the first calf developed fatal mucosal disease soon after inoculation with BVD-TGAC virus. The second

calf had mild self-limiting illness and the third calf remained healthy. It was concluded that the humoral response may not play an important role in the pathogenesis of mucosal disease. Other factors of the host-viral relationship that should be investigated for a possible role in mucosal disease include the virulence of the cytopathic virus; the interaction between the cytopathic and noncytopathic virus; and the host's cell mediated immune response to the virus(es).

The second study provided strong circumstantial evidence for a pathogenic role of cytopathic virus in spontaneous chronic BVD. Four cattle with chronic BVD had lesions reminiscent of mucosal disease and cytopathic virus was isolated from three of these cattle. The hypothesis that chronic BVD is caused by cytopathic viral superinfection of persistently infected cattle[25,90] awaits experimental verification.

Cytopathic superinfection of persistently infected cattle may form a spectrum of disease. A chronologic scale of cytopathic superinfection would have acute mucosal disease at one end and chronic BVD at the other end. Except for one heifer with spontaneous mucosal disease, all of the cattle with mucosal disease could be assigned to the acute end. These cattle had diarrhea for less than a week and had acute gastrointestinal and lymphoid lesions. The one exception, No. 4, had diarrhea for a month and had subacute lesions typical of mucosal disease. She would be placed in the border zone between acute mucosal disease and chronic BVD ("subacute mucosal disease" or "subacute BVD"). Three of four cattle with chronic BVD would be assigned to the middle of the chronic BVD region. These cattle had subacute to chronic partially healed gastrointestinal

lesions and two cattle had clinical enteric dysfunction exceeding two months. One cow (No. 13) would be assigned to the far end of the chronic BVD region. She had diarrhea for six months and had chronic fibrotic lesions of the large intestine. Of all the cattle with intestinal lesions, No. 13 was the only individual from which cytopathic virus was not isolated.

A consistent lesion in cattle with mucosal disease and chronic BVD (except No. 13) was multifocal mucosal necrosis of the upper alimentary tract. This lesion was not found in cattle with persistent and induced acute BVD viral infection. Both viral biotypes may have to be present in the upper alimentary tract mucosa to induce these necrotic lesions typical of mucosal disease.

A consistent lesion in calves with induced acute BVD viral infection was acute tracheitis. Viral inoculation was by the intranasal route. The tracheal lesion could have resulted from direct aerogenous viral infection of the trachea or from hematogenous viral spread to the trachea during viremia. An alternate hypothesis is that secondary bacterial colonization of the trachea induced the lesion.

Acute BVD viral infection in young cattle may play a synergistic role in bovine respiratory disease complex (shipping fever pneumonia). This syndrome of feedlot cattle is a mixed respiratory infection involving Pasteurella haemolytica serotype 1 with other bacteria and viruses. In some reports, BVD virus was the virus isolated most frequently from feedlot cattle during shipping fever outbreaks[27,121]. Experimental mixed infections of BVD virus with Pasteurella haemolytica[111,113]

indicated that BVD virus may enhance the pathogenicity of P. haemolytica. Besides inducing immunosuppression,[110,122,131,132] acute BVD viral infection may contribute to bovine respiratory disease by eliciting necrosis and inflammation of upper respiratory tract mucosa, permitting bacterial colonization.

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